

Setting the Soviet academy free

The Academy of Sciences of the USSR has suddenly been declared free from external influence. This is a welcome development, but one full of danger.

THE most striking feature of the report on page 111 from our Moscow correspondent, Yuri Kanin, about the new status of the Soviet Academy of Sciences is the language that he uses. In a few months, Moscow and the rest of the Soviet Union has accepted that the independent republics are destined to operate autonomously, obligated to the centre only for the services they agree should be provided centrally — international defence for example. Although the legislation that will define this new relationship has yet finally to be negotiated (between the centre and the republics) and carried by the Supreme Soviet, people write and talk as if it were already a reality. This a far cry from the period earlier this year when the Baltic republics were nervously making a case for autonomy. (In the past few weeks a new government in Armenia has not merely followed suit, but has defeated an armed protest in part inspired by the Communist Party's local officials.) The decision that the all-union academy should also be autonomous is an adaptation, this time by the centre, to the same process of change.

Matters of nomenclature are important, but also confusing. This journal has never knowingly mistaken Russia for what it has called the Soviet Union, but Mr Boris Yeltsin's Russian Federation is the driving force for this latest stage of *perestroika*. That seems also to be the reason why a change in the status of the central academy had to be put in train. It is not surprising that most of the academy institutes are sited in Yeltsin's Russian Federation which, stretching from Leningrad to Vladivostok, is a half of the Soviet Union by area and perhaps two-thirds by sophistication and even wealth. But the Russian Federation differs from the other 15 republics in not having an academy of its own. If the status of the "big academy" had been left unchanged, Yeltsin would have made a grab for it. Even now, but not immediately, there is a high chance that he will acquire some parts of it.

Meanwhile, there is good news and bad news. It is thoroughly to be welcomed that the Soviet academy will now be free from government and even party interference. Among other things, it will now be able to run its own affairs, sending to meetings overseas the people whom the organizers have invited and promoting members of its own institutes on the reputation of their work without political (or even ethnic) calculation. But even this will not be simple. The Soviet government has

decreed independence, but it has not yet said whether the academy will have a budget big enough to keep able people away from the fleshpots of the West, let alone to keep on its books the even larger battalions of those who have become inactive. Nor can it until there is a union treaty between the centre and the republics to determine which governments own what.

Balance

More immediately, the Soviet academy will have to decide how to balance its functions of being a focus for excellence in research and scholarship with its public role of managing a substantial part of the union's research. The wise course is to separate these functions, and quickly. But the academy will also have to show more courage than in the past 60 years (when Stalin made it move from Leningrad to Moscow) if it is to prove to the world outside that it has a voice of its own, not just an echo of the government's. It will not readily be forgotten that the academy, while not expelling Sakharov, did its best to ostracize him.

The bad news is of two kinds. First, the independence now granted to the academy is like that of a man imprisoned in the middle of a desert whose jailers one day say that he is free to go. Go where? While the Soviet economy remains on its knees, there is a danger that even an adequate budget would not allow it to meet its institutes' need for up-to-date equipment and their employees' needs for the necessities of life, housing for example. And while the central committee may no longer have a right to interfere, it will be harder to shake off the party's influence at the laboratory level. Yet there are enough able and adventurous people in the academy for them, by good management and the judicious use of the freedom to form cooperatives, to provide an example for the rest of the Soviet Union. Will they be given the encouragement, and will they have the courage?

It is also bad news that, by giving the Soviet academy independence, the Soviet government may be tempted to believe it has made a lasting policy for science. Let us hope it does not cherish this illusion. For the past 70 years, science has been at the centre of Soviet government policy, and the Soviet academy has been the chief instrument thereof. The academy has loyally bent its interests to match the ambitions of the government, but usually



ineffectually. Yet simply by being in existence, the academy has drained people and support from the universities, leaving it to republic academies to redress the balance in respect of universities in their own territory. (Moscow University is the exception.) But that has always been an awkward solution, depending on the ability and willingness of academy institutes to provide bright young people with a training in research. If the Soviet academy is to be independent, and also under serious budget pressure, there will have to be a complementary policy on universities as part of the union treaty. Let us hope that, in the scramble to recreate the Soviet Union, that is not forgotten. □

Counting candle-ends

The British Treasury, habitually parsimonious, sacrifices sovereignty in getting its pound of flesh from Brussels.

THE British Treasury has a well deserved reputation for being mean dating at least from its practice, in Gladstone's time, of regarding as a national asset the Civil Service's stock of unspent candle-ends. The tradition is splendidly enshrined in the Treasury's attitude towards research grants offered by the European Commission under the collaborative research programmes of the European Community (EC). The tortured logic, linked with the Eurospeak concepts of "additionality", "attribution" and "substitutability", is laid out admirably in this summer's report on EC research from the House of Lords Select Committee on the European Communities (see *Nature* 346, 305; 1990). Attribution turns out to be the Treasury's way of subverting additionality.

This is how the argument goes. Suppose some British organization applies for and is awarded a grant from the EC. The grant will be made from funds provided by member states, of whom Britain is one. The EC makes grants on the understanding that they will have a "genuine additional economic impact". Although devised to regulate spending from the EC social fund, this principle of "additionality" is supposed to apply to all community grants. So what happens when British applicants win EC grants? Somebody at the Treasury "attributes" the funds received from Brussels to whichever government departments might have incurred that spending. And then, in the following year, depending on an assessment of the "substitutability" of Brussels for British money, the budgets of the government departments concerned will be reduced accordingly.

The principle of additionality is not formally breached. EC grants are indeed spent on the purposes intended by Brussels. All that happens is that government departments' capacity to spend money on similar projects is reduced in later years. Attribution seems not yet to have begun to bite on British spending on research, but the Science and Engineering Research Council, in its evidence to the House of Lords committee, was alarmed

that a Brussels grant of more than £1 million to equip its Neutron Spallation Source to generate muons (to be used by groups from elsewhere in Europe) will be offset by a corresponding hole in its budget.

How can this procedure be defended? The Treasury's argument is that it is one thing to have to pay Britain's annual contribution to the EC (which is a money transfer) but quite another, and worse, to see some of that money return to finance unplanned public expenditure, to the control of which it has long been dedicated. The argument that all public spending engenders inflation may not apply to external sources of funds as it does to the domestic variety, but the true folly of attribution is that it is a way of substituting Brussels's priorities for the British government's. When the British fear as they do the sacrifice of sovereignty to Brussels, the continued practice of attribution seems perverse as well as mean. □

Fusion delayed

The joke that budgets for fusion research are inversely correlated with oil prices (see page 114) is not funny.

WILL there ever be a working thermonuclear fusion reactor, and if so, when? The question is more than academic when governments are casting around zealously for alternative sources of energy. The increase of the world price of crude oil following Iraq's annexation of Kuwait does not imply that supplies of oil are coming to an end, but supplies of oil at \$30 a barrel have dried up, at least for the time being. It is natural that governments worrying about their support for windmills and uranium-driven nuclear power should now think more fondly of thermonuclear fusion as well.

The plain truth, sadly, is that there is not much to go on. The fuel (deuterium) may be plentiful and reasonably cheap, but the capital costs of building thermonuclear power stations are essentially incalculable. The international project (INTER) to design a large prototype (see page 114) should provide some kind of yardstick, but even that will not accurately foretell what maintenance costs will arise from the need to replace parts regularly exposed to intense radiation or blobs of hot plasma (as in magnetic-confinement machines). Only operating experience will do that. And then there are the imponderables arising from the need somehow to absorb neutrons produced in fusion, and the public anxiety that will attend the disposal of radioactive waste.

That is why there is the strongest possible case for pushing ahead with the design study, and the construction that should follow. The objective, at this stage, should not be to generate huge amounts of power, but simply to estimate the economic parameters of these machines. But this time governments should not relax when the price of oil falls back below \$20 a barrel. Whatever the outcome of the present troubles in the Middle East, fluctuating prices have come to stay. □

Draft of conflict rules angers Congress

- Weiss sees backsliding in the Public Health Service
- Universities police themselves

Washington

THE battle between the research community and the US Congress over conflicts of interest in research seems about to be rejoined. That, at least, seems the purpose of Representative Ted Weiss, chairman of the House of Representatives Government Operations Committee, which this week released an internal draft of proposed federal regulations on conflicts of interest in research, as well as the results of its own three-year investigation of the problem.

The congressional report, citing case studies of improper university and government handling of research conflicts, says that institutions are reluctant to find fault with their own researchers, even retaliating against whistleblowers. The report also says that federal funding agencies, in particular the National Institutes of Health (NIH), have repeatedly ignored problems arising as well as their own principles on conflicts of interest.

The draft federal regulations that the committee has now made public show that the government is planning substantially to weaken the regulations it had earlier proposed, in part because of a backlash from researchers who complained that the rules were unnecessarily intrusive.

The draft regulations, dated 16 July this year, differ from previous versions in that they apply only to clinical trials supported by the Public Health Service (PHS), which includes NIH. Other PHS-supported research will be subject only to whatever rules exist at the institution at which it takes place. An accompanying memorandum by PHS regulation officer John Gallivan explains that the focus on clinical trials arises from "the near-term significance of this kind of research" and its ties to commercial products.

Universities and other research institutions will determine whether conflicts of interest exist among their researchers. "Designated institution officials may approve the ownership or acquisition, by investigators participating in PHS-supported clinical trials, of financial interests in organizations that manufacture, sell or otherwise have property rights in the product under study. But "such interest must be judged unlikely to compromise the design, conduct or reporting of the ... research", say the draft regulations.

Previous versions of the regulations stipulated that researchers "shall not have financial interest" in such organizations un-

less the interest "is judged so small as to rule out any significant likelihood" that the outcome of research would be distorted.

In a statement, Weiss attacked the draft PHS regulations as a step in the wrong direction. The regulations "do not suggest the level of commitment by the federal government that will be needed" to resolve problems of the kind documented in his own committee's report, Weiss says. "The net effect of promulgating such regulations would be to maintain the *status quo*", he argued.

Last year, PHS proposed strict conflict-of-interest guidelines to ensure a uniform set of standards at all PHS-supported institutions. But universities protested that compliance would require enormous amounts of paperwork for no clear benefit. PHS retracted the guidelines early this year (*Nature* 343, 104; 11 January 1990), promising to produce a less onerous set of legally binding regulations. The draft released by Weiss's committee this week is thought to be close to the final rules that will be published later this year.

Weiss is concerned that the new regulations require researchers to disclose their financial holdings only to their own institution's administrators, rather than directly to the federal agencies or the general public. Pointing to the examples of improper and ignored conflict of interest documented in the committee report, one congressional staff-member notes that "these administrators are the same people who are telling us that there is no problem".

The long-awaited congressional report on the scope of the problem documents ten examples of the failure of existing safeguards against conflicts of interest. Some of the cases have already received attention, but others have not previously been made public. Among the cases, the report details the financial holding of researchers studying the efficacy of TPA, the clot-dissolving drug manufactured by Genentech. At least 14 of the researchers involved owned Genentech stock, a fact they had not initially revealed.

The report also notes that although the researchers reported that TPA performed better than its competitor (streptokinase), later independent studies showed that neither drug was substantially superior to the other, and both had dangerous side effects.

Also documented is the case of Retin-A, an acne cream that has recently been sold as drug to reverse wrinkling — a claim

SPACE TRANSPORT

Piggy-back ride for an interim Hotol?

London

BRITISH Aerospace's Hotol spaceplane project, which has been floundering ever since the British government decided to withdraw funding two years ago, may be back in business with a little help from the Soviet Union. A new plan calls for the plane to be carried high into the atmosphere riding piggy-back on an Antonov An-225 — the world's largest aircraft.

Hotol was conceived as a revolutionary unmanned reusable space launch vehicle, capable of taking off from a horizontal runway powered by an air-breathing rocket engine. But the engine's development costs, accounting for more than half of the £6,000 million project, have proved prohibitive.

British Aerospace is now planning to leave the development of the engine until later. A six-month study with the Soviet aviation ministry will see if Hotol could be launched from the back of the Soviet Antonov An-225 transport aircraft and powered into space by conventional rockets, perhaps supplied by the Soviets.

The An-225 was designed to carry large structures on its back, and has been used for transporting the Soviet space shuttle. But to launch Hotol into space, the An-225 may need an engine upgrade, to carry the heavy aircraft and its payload to about 9 km altitude. British Aerospace calls the new plan the 'interim Hotol', and hopes that the original concept, complete with the air-breathing engine, can be developed at a later date.

If successful, the interim system could offer 7-tonne payload launches for \$10–12 million by early next century — about a quarter of the price of launches on the latest version of the European Space Agency (ESA)'s Ariane rocket. A British Aerospace spokesman says that ESA and other agencies will need cheap replacements for existing launchers, to put the many planned Earth observation and communications satellites into orbit. Peter Aldhous

based on research published in the *Journal of the American Medical Association* in 1988. After other scientists disputed Retin-A's effectiveness as an anti-ageing drug, the original researchers admitted that the *J. Am. med. Ass.* study had not been double-blind.

Investigations by the Weiss committee are said now to have revealed that several of the original researchers had served as spokesmen for Johnson & Johnson, Retin-A's manufacturer. Others had received Johnson & Johnson honoraria and research grants. A recent NIH conference on the ageing effect of light concluded that the safety and efficacy of Retin-A has not been established, the report says.

Christopher Anderson

Asking for the Moon . . .

Tokyo

ASTRONOMY and space science come out winners in the budget request for science submitted by Japan's Ministry of Education, Science and Culture (MESC) at the end of last month.

The budget request will be whittled down in negotiations with the Ministry of Finance before the end of the year, but is unlikely to suffer major change. The key negotiations over the budget occur internally within MESC before submission to the finance ministry.

As expected, MESC has at last decided to give full backing to a project to build one of the world's largest optical infrared telescopes in Hawaii (see *Nature* 346, 686; 1990). The ministry has applied for ¥650 million (\$4.6 million) for equipment for the telescope and ¥13 million for other expenses. Construction is expected to take eight years.

Under the original plan proposed many years ago, the single-mirror telescope was to have had a diameter of 7.5 metres. But a study this fiscal year has shown that present-day technology is capable of making a larger mirror, so the size has been increased to 8 metres. This will make the Japanese telescope one of the largest in the world (a 10-metre optical infrared telescope is now being constructed by California Institute of Technology in the United States).

On the other hand, MESC fears that the Ministry of Finance will balk at last year's estimated cost of ¥45,000 million (\$320 million) for the telescope, so the projected cost has been reduced to ¥38,000 million by including only "essential facilities", according to Makoto Kinoshita of MESC's research institutes division. Not so lucky is a competing proposal to build the world's largest observatory for neutrinos and proton decay (see *Nature* 331, 379; 1990). The observatory, called Superkamiokande, would be built in a mine in Gifu Prefecture. But, contrary to the expectations of some supporters of the scheme, MESC has decided not to apply for specific funds for the project next year. Rather, it hopes to find some money for excavating a hole for the observatory from general construction funds which will not be allocated until March next year. Until then, the future of the project remains uncertain.

MESC has decided to go ahead with a second mission to the Moon proposed by the Institute of Space and Astronautical Science (ISAS) as a follow-up to this year's successful lunar launch (see *Nature*

344, 693; 1990). The ministry has applied for ¥400 million (\$2.9 million) to start development of the lunar satellite expected to be launched in early 1996; it will drop seismometers onto the lunar surface to monitor "moonquakes". The budget for space also rises because of increased development costs for a new and larger solid-fuel rocket (M-V) that ISAS is developing for the lunar mission and missions to the planets.

The increased support for astronomy and space science is affecting other parts of MESC's overall budget for science because of strict budget limits laid down by

MESC BUDGET REQUEST

	¥000 million	% Change from 1991
Grants-in-aid of research	59.0	+ 5.7
Government/industry research	13.4	+16.5
Donations from industry	44.3	+13.0
Nuclear fusion	8.8	- 1.8
Accelerator physics (TRISTAN)	15.8	- 2.7
Space science	21.3	+18.2
Astronomy	1.7	+95.3
Earth science	2.3	+ 1.4
Antarctic research	3.3	-35.8
Domestic research fellowships	2.4	+12.3
International exchange	6.7	+ 8.7

the Ministry of Finance. Among the projects to suffer is TRISTAN, the giant electron-positron collider at the High Energy Physics Laboratory (KEK) in Tsukuba. Operating hours for the accelerator, which runs up huge electricity bills when switched on, will be reduced by 100 hours to 2,800 hours in 1991. TRISTAN was recently criticized in the influential economic newspaper, the *Nihon Keizai Shimbun*, for failing to reach its goal of finding the top quark. And some senior Japanese physicists at TRISTAN have left for other jobs.

Some scientists are pressing MESC to convert the main ring of the collider into the world's largest synchrotron (offering energies of up to 10 GeV). But Kinoshita says TRISTAN still has many important scientific investigations to carry out and MESC intends to continue supporting the accelerator for at least the next few years.

David Swinbanks

Correction: Animal Welfare Institute

A SENTENCE in the 30 August article on US animal regulations entitled "One step forward . . ." (*Nature* 346, 782; 1990) incorrectly paraphrased William Cotreau of the Animal Welfare Institute on the frequency of inspections of dog dealers. Federal officials were unable to inspect one-quarter of all dog dealers in 1988, not three-quarters, as the article implied. □

Budget brings a little cheer

Sydney

EDUCATION came out a winner in the Australian federal budget announced at the end of August, making it almost certain that significant pay rises are on the way for academics. Scientific research did less well overall, having received a major boost in last year's Science and Technology Statement.

Until now, salaries for academics have been "insulated from wage movements in the rest of community", according to Wayne Burns of the Department of Education. But thanks to the budget increase, salaries will be set by the Australian Industrial Relations Tribunal in line with wages in other sectors. Junior academics and postgraduate teaching assistants are expected to benefit most. Graduates will lose from changes — they will be expected to repay their fees more quickly after they have graduated and obtained jobs. The Commonwealth Scientific and Industrial Research Organization (CSIRO) came out at a net loss. Although given an extra A\$4.1 million for new projects in addition to an increase of A\$30.1 million won under a three-year agreement, CSIRO also lost A\$3.8 million as part of a plan to promote efficiency in the organization. As a result, "existing activities will be cut in real terms", says Lindsay Bevege of CSIRO.

A key element of the budget is funding to establish 50 Cooperative Research Centres over the next five years. The centres will be located next to universities and will receive matching funds from state governments and industry. The budget allocates A\$4 million for 15 centres in 1990-91.

Medical research funding provided few surprises, with the National Health and Medical Research Council receiving A\$99 million for next year. But a new capital works programme costing A\$45 million over five years was introduced. Institutes will have to compete for funds and their bids will need to demonstrate "a potential for commercial exploitation of research results", according to Brian Howe, the Minister of Health. Once again, matching funds are expected from state governments and industry.

AIDS funding received a significant boost with an extra A\$78.3 million for 1990-91, including a 47 per cent increase to A\$47.2 million for AIDS education, prevention and support services. AIDS research funding rose by 52 per cent to A\$7.6 million but researchers are unhappy that the government did not provide a A\$500,000 increase to compensate for inflation which was promised last year.

Tania Ewing

Academy to stand alone

Moscow

WITH a surprise decree, President Mikhail Gorbachev has declared the Soviet Academy of Sciences a self-governing organization working on the basis of Soviet law and its own statutes.

Now, no state, party or other organization will have a right to interfere in the affairs of the academy, which will own all the fixed assets under its control, including institutes, laboratories and enterprises. State financing of the academy's research will continue, but the decree requires the results to be transferred to the state under appropriate copyright arrangements.

The granting of independence to the academy and its separation from the government was welcomed by Academician Yuri Osipyan, an academy vice-president and a member of the presidential council. He says that, in the past, the USSR Council of Ministers has unduly influenced the academy's management. (The same might be said of the Central Committee of the Party.) Often, as in the suppression of genetics and cybernetics, and in the criticism of "wilful" scientists for elitism, the academy has been over-obedient to higher authorities.

The academy is now freed from administrative bonds, but is faced with the challenge of how to use its independence.

The decree has nevertheless met with a mixed response from the scientific community, at least in part because there had been no prior discussion of it. Some fear that the decree will entrench the monopoly of the academy in research and confirm in its present position a management now much criticized from below. There are also fears that the democratization of the academy will be impeded.

Some also ask whether the hidden purpose of the decree is to prevent academy institutes on the territory of the Russian Federation from falling into the hands of the proposed Russian Academy of Sciences, which has been much discussed in the Soviet press. Alone among the republics of the Soviet Union, the Russian Federation anachronistically lacks an academy of its own.

There is powerful support for the founding of a Russian academy. In an interview, Nicolai Laverov, chairman of the USSR State Committee for Science and Technology and vice-chairman of the USSR Council of Ministers, said that "there must certainly be a Russian academy, but not at the cost of the destruction of the all-union academy".

Laverov nevertheless believes that there are several strong institutes on Russian soil that could be the foundation of a Russian academy, carrying out fundamental studies and dealing with specific republic problems. He notes

that the new decree refers to an all-union research fund from which studies by republic academies will be supported when they are part of a national plan. The proposed Russian academy, he says, should have the special task of engaging in the research enterprise scientists now working in higher education.

The proposed research fund is an important innovation. According to Laverov, Both the Soviet Union (with the consent of the Supreme Soviet of the USSR) and the union republics will contribute to it. There will be a governing council representing the union and republic academies, Laverov's state committee and higher education and industrial institutes engaged in basic research, which means that academy institutes will not be the only beneficiaries. Laverov says that there will also be target-orientated funds for the support of research.

Laverov does not rule out the possibility that some republics will refuse to contribute to this all-union research organization. Some of them, he acknowledges, will find it difficult to contribute, for which reason the general budget of the Soviet Union (based on taxation of enterprise profits) will remain the foundation of fundamental research.

But many academy scientists are worried by the money problem. The capacity of the centre to finance research will depend on the power it retains under the union treaty now being negotiated.

Meanwhile, the presidential decree will offer all-union scientists a pay rise as well as a contract system of employment, both within the Soviet Union and abroad. These provisions seemed to have been prompted by the expected law on free emigration from the Soviet Union, and the potential brain drain that may follow. Poor equipment and working conditions, and miserably low pay, are forcing the more talented young researchers to think of leaving for research centres in the West.

Laverov says the emigration law will certainly bring "major difficulties" for science, which is why the new decree offers means by which the living and working conditions of academy staff may be improved. He says that the contract system should allow for faster promotion and the timely recognition of talented scientists. He notes that the contract system will impose obligation on scientists as well as employing organizations.

"Nobody particularly intends to create obstacles for Soviet scientists to work abroad", he says, "but the contract system is meant to ensure civilized conditions for the process". It will also regulate the work of foreign researchers at institutes of the Soviet academy.

Yuri Kanin

Novosti, Moscow

Death strip becomes nature preserve

Munich

DESPITE its legendary lack of concern for the environment, the former Communist regime in East Germany did inadvertently create one massive nature reserve — the 600-km-long, 5-km-wide 'death strip' that separated East and West Germany. The strip, and the huge hunting estates kept by Communist party bosses, provided a safe home for rare species of cranes, storks, whitefish and wild flowers. But peace and prosperity are now threatening these creatures and their habitats as prosperous West German tourists pour over the border into East Germany.

Environment officials in both parts of Germany have responded with a controversial attempt to create nature preserves along the former border. West German Environment Minister Klaus Töpfer declares that the "green belt" along the border is "the only positive thing to emerge from the division of Germany". But residents complain that the creation of parks will continue the isolation imposed by the impossibility of crossing the 'death strip'. The mayor of the East German village of Lassahn protested to the West German newspaper *Frankfurter Rundschau*, "Why should we drive 90 kilometres to the next town when it is only 12 kilometres away?" A spokeswoman at the Bonn Environment Ministry says that, since the borders opened, thousands of tourists from both parts of Germany have picnicked and hiked all over the more popular border areas such as the Brocken in the Harz Mountains. Although Bonn does not plan to stop them from going there altogether, "we are afraid that the tourists will soon trample everything into the ground" if the government does not step in soon.

The black stork *Ciconia nigra* and the crane *Grus grus* are endangered by unification, says Knut Haarmann of the West German Research Centre for Nature Protection in Bonn. Only through the efforts of ecologists, who have erected protective shelters in remote areas, have 50 breeding pairs survived. But many hundred pairs of each exist in East Germany.

Huge collective farms dotted with uncultivated, swampy patches, which are ideal for breeding and the raising of young, have also contributed to the survival of black storks and cranes in East Germany, says Haarmann.

It will be up to the *Länder* (states), not the federal government, to create nature preserves in the areas in question, but Fritz Dieterich of the Bonn environment ministry said that Bonn will go as far as possible to coordinate such efforts. Although not every area may be protected, he says, "on a large scale, I predict we will succeed".

Steven Dickman

A one per cent solution?

San Francisco

THE US semiconductor industry's leading corporations and a self-employed engineer, Gilbert P. Hyatt, are getting ready for multimillion dollar negotiations following last month's announcement that the US Patent Office has awarded Hyatt a patent covering virtually every microprocessor in use today. The patent runs from its effective filing date of 1970, pre-dating the claim from the Intel Corporation team generally credited with creating the microprocessor. An Intel spokesman said last week that they are reviewing the patent, but have yet to decide its impact on the company or whether Intel would challenge it.

Hyatt's strategy is crucial. Because patents are not self-enforcing, Hyatt will have to take on the semiconductor makers in order to receive payment. Last week, Hyatt claimed some early successes, saying that he has nearly completed licensing fee negotiations with one major electronics corporation and that several other companies are already waiting to do business with him. Although he will not name the amount of money he is asking, Hyatt says he is looking for "a reasonable royalty that won't be a burden".

But even modest fees may add up to large amounts of money if the patent's validity withstands legal challenge. Ironically, if questioned in court, the patent may benefit from the 20 years it spent under review. Lawyers will now find it

hard to make new arguments but may attempt to narrow the patent's coverage to more specific types of microprocessors, or argue that Hyatt's claim is invalid because he did not actually transform the technology into working products.

Royalty fees in the semiconductor industry are typically one to five per cent of a company's revenues on that product.

According to Willis E. Higgins, a patent attorney familiar with the document, Hyatt is likely to ask for a fee of less than one per cent in hopes of avoiding a litigious response from the corporations. Even so, the inventor stands to become a multimillionaire. In addition to US claims, Hyatt also has the right to seek royalties from foreign companies for the microprocessors that they make, use or sell in the United States.

Hyatt developed his microprocessor at a small company called Micro Computer, Inc. that he founded in 1968, then dissolved in 1971. The engineer, who holds over 50 other patents, supports his research through consulting for the aerospace industry. This is his first profitable patent, and he says he plans to channel its proceeds into his current personal computer research, which involves a radical departure from current technology. As he matter-of-factly describes it, "Twenty-two years ago I developed the PC for the twentieth century. Now I'm working on the PC for the twenty-first century."

Elizabeth Schaefer

COMPUTER INDUSTRY

ICL deal threatens research links

London

THE British computer company ICL's continued involvement in collaborative European research projects is in question, thanks to the disquiet of other European companies over the sale of an 80 per cent holding in ICL to the Japanese company Fujitsu. The board of JESSI (the Joint European Submicron Silicon Initiative) will meet on 27 November, three days before the Fujitsu deal is finalized, to decide if ICL can remain a member.

JESSI was set up by a group of European computer companies to promote collaborative research on the fabrication of semiconductors, and so enable the European industry to compete with the United States and Japan.

ICL is not heavily involved in JESSI projects, as the company obtains semiconductors from Fujitsu. But a spokesman rejects the accusation that ICL will become an outpost of the Japanese industry, not entitled to participate in European projects: "We continue to regard ourselves

as a European-based company". He adds that the JESSI committee with which ICL is most strongly connected, concerned with applications for semiconductor materials, has said that ICL should not be expelled.

The European Commission seems to be taking a softer line over ICL's involvement in some 40 projects within its ESPRIT information technology research programme. In any case, US companies already participate in several European Communities research projects.

Filippo Pandolfi, Commission vice-president in charge of research, has said that projects must be reviewed case by case and that it will be for the partners involved in each to decide if they wish to continue collaborating with ICL.

JESSI officials also have a more immediate concern than the ICL question — last month's decision of the Dutch electronics company Philips to pull out of JESSI's advanced memory chip project, which accounts for about 20 per cent of JESSI spending.

Peter Aldhous

The long and winding road

London

As the attention of the world's environmental diplomats switches to negotiating a climate change convention, there seems to be a broad international consensus that the task must be finished in time for the United Nations conference on environment and development, due to take place in Brazil in 1992. But whether the convention should be an agreement with substantial measures to combat global warming or a bare-bones statement of concern is more contentious.

Now the Intergovernmental Panel on Climate Change (IPCC)'s report has been completed (see *Nature* 347, 9; 6 September 1990), the climate change circus moves to Geneva on 24 September, where government representatives will lay down the ground rules for the negotiations proper. These start in Washington in February 1991, at a meeting hosted by US President George Bush.

William Nitze, from the US Alliance to Save Energy, who helped begin the IPCC process during his time at the US State Department, believes anti-global-warming measures must be incorporated into the initial convention: a more general agreement "just won't cut it", he says. West Germany and the Scandinavian nations are likely to back this position, but the current generation of US negotiators will aim for an agreement along the lines of the 1985 Vienna Convention, which simply stated international concern over the thinning ozone layer. Firm targets on emissions of ozone-depleting chemicals were agreed only later, under the 1987 Montreal Protocol.

Stewart Boyle, from the UK Association for the Conservation of Energy, says that in the case of climate change there is no need for a two-stage process of convention followed by protocol. The Montreal Protocol had to wait for scientific review, he says, which in this case IPCC has already provided. But US determination to stress the uncertainties surrounding current climate predictions, despite their failure to water down significantly the conclusions of the IPCC report, may force negotiations into two stages.

The British negotiating team is preparing for an 'honest broker' role. The United Kingdom is one of relatively few countries so far committed to stabilizing carbon dioxide emissions (see *Nature* 345, 373; 31 May 1990), provided other countries play their part. But UK negotiators are determined not to let calls from some countries for the inclusion of rigid emission targets scupper the delicate convention negotiations.

Nitze says that the option of a more

substantial convention could be kept open almost until the last minute, by keeping controversial sections 'bracketed off' from the main text, to be included if there is a change of heart by the United States. He believes that some measures, including clauses on deforestation, strengthening climate monitoring and defining financial arrangements to help poorer countries to develop their economies while minimizing greenhouse gas emissions, may well be included.

The inclusion of carbon dioxide emission targets (themselves an interim step towards integrated controls encompassing all greenhouse gases) is far less likely. But the United States may find itself forced into conceding some targets if many nations produce their own emissions strategies before 1992. The positions of individual countries will become clearer during the two-day ministerial meeting which concludes the World Climate Conference in Geneva in November. The urgency with which the international community wishes to address climate change convention negotiations should also be evident in the meeting's final declaration.

IGBP GLOBAL CHANGE PROGRAMME

Stripped for action

Paris

AFTER two years of discussions, the special committee of the International Geosphere-Biosphere Programme (IGBP) on global change met last week in Paris to discuss details of a set of five honed-down 'core projects' which it expects governments to back over the next 10 to 20 years. Although data from these first projects are seen as essential if politicians are to make informed decisions on global change issues, IGBP officials are still concerned that not all governments are fully committed to the programme. A special problem will be the need to gather information from parts of the globe occupied by under-developed nations that lack their own research capabilities.

IGBP was put together by the International Council of Scientific Unions (ICSU) in September 1986 and, with the World Climate Research Programme (jointly sponsored by ICSU and the World Meteorological Organisation), is the coordinating power behind the international effort to understand global change. United Nations backing came with a General Assembly resolution passed in December 1989.

The Paris meeting exuded an air of self-congratulation, as though a difficult job had been well done. According to Marie-Louise Chanin, one of the 19 members of the IGBP special committee, any project not judged immediately relevant to a list of seven critical questions on global change was eliminated over the course of

European Communities (EC) partners failed to reach a common position on carbon dioxide emissions when ministers last debated the issue in June. Spain, showing little interest in the problem, is the main stumbling block. But another meeting is planned for 29 October, and it is possible that a general EC target can be set, within which Spain and the other southern European nations have some leeway. The slack could be made up by EC member states already committed to stronger action, such as the Netherlands and West Germany.

A firm emissions target from Japan will be influential, since the Japanese have been seen as part of the camp opposed to emissions targets which is headed by the United States and backed by the Soviet Union and Saudi Arabia. Japan has reasonably complained that, as the most energy-efficient nation in the developed world, it has already done more than most to address the emissions problem (see table). But Japan is expected to announce during October a plan to stabilize its emissions by 2000, albeit at a level marginally higher than today.

Peter Aldhous

meetings involving more than 500 scientists. The five surviving projects will look at global atmospheric chemistry, global ocean flux, biospheric aspects of the hydrological cycle, terrestrial ecosystems and global changes over the past 2,000 years. Several of these projects have already started.

IGBP now has 46 member nations but, says French Research Minister Hubert Curien, "there are some serious gaps, particularly in South America, Africa and Oceania". The problem for poorer countries, says Dr E. Diop of Senegal, is that they tend to be submerged by immediate problems such as drought and famine, and do not look for long-term causes and effects. IGBP now hopes to overcome this isolation by creating a series of regional research centres in developing countries. These will concentrate attention on synthesis and modelling projects particularly relevant to regional priorities, while providing opportunities for training and exchange.

Now that the planning phase is complete, said John Wood, of the marine sciences directorate at the UK Natural Environment Research Council (NERC), IGBP must be implemented. Core projects will be funded through national research agencies, with coordination ensured by 'core project offices' dotted around the world. So far, offices have been set up in Kiel and Berlin (Germany), Berne (Switzerland) and Paris. The Paris office, to be located at the University of

Room for improvement on emissions

THE table shows 1987 greenhouse gas emissions for a number of key countries, compiled by the World Resources Institute (WRI). Emissions of methane and chlorofluorocarbons have been converted to quantities of carbon dioxide with the same heating effect, and the figures include the effect

	GREENHOUSE GAS EMISSIONS (TONNES OF CARBON EQUIVALENT)	
	Total (millions)	Per capita
US	1.00	4.1
Soviet Union	0.77	2.7
European Communities	0.76	2.3
Brazil	0.47	3.3
China	0.44	0.4
Japan	0.23	1.9
W. Germany	0.17	2.8
UK	0.15	2.6

of deforestation on the carbon dioxide budget. The total emissions figures identify the main 'climate polluters', says Allen Hammond from WRI. He believes the ultimate aim of an international agreement should be to reduce emissions from these countries. When Germany is reunified, European Communities emissions will exceed those of the Soviet Union. But any agreement must also take account of the varying extent to which countries are able to make reductions. The per capita figures show, for example, that US emissions per head of population are more than twice those of Japan. If Japan is taken as a model energy-efficient economy, it seems that there may be scope for reducing greenhouse emissions in the US and to a lesser extent in the Soviet Union and the European Communities. Emissions targets based on per capita figures would also benefit developing countries such as China, with low per capita emissions. The high per capita figure for Brazil is explained by the high rate of deforestation in that country in 1987.

Peter Aldhous

Paris VI (Jussieu) will house the IGBP data and information system directory. Co-funded by the US National Aeronautics and Space Administration (NASA) and the French government, the office will serve as a clearing house for Earth observation satellite data, ensuring compatibility and links with other databanks. The centre will be directed by S. Ichtiague Rasool, scientific director at NASA in Washington, from 1 October.

One of the goals of IGBP is to be able to make long-term predictions of climate change. Woods thinks that this will be feasible in 20 years, but that a start needs to be made "in the next 2-3 years".

According to Woods, "if governments do not take this decision (to implement IGBP), they will have consciously decided not to base their policies on the best available predictions". Funding could be a problem, despite the political enthusiasm for global change research. This year, IGBP fell \$500,000 short of its \$1.6 million target budget for administration.

Peter Coles

Fusion's turn again?

Washington & London

AN old rule of thumb among US fusion researchers holds that the funding for the field almost always parallels the average price of oil, delayed by about a year. Although the current budget crisis may put that rule to the test, signs are emerging that political support for fusion research in the United States and Europe is strengthening again as oil prices rise.

This week, two reports from US panels will join a just-released study from the European Communities (EC) in setting priorities for fusion research in the coming decades. Representatives from Japan, the EC and the United States will discuss the new findings at a meeting in Washington later this month.

One US panel — a Department of Energy (DOE) independent advisory body led by H. Guyford Stever — will recommend that DOE set goals of a demonstration fusion power plant by 2025 and an operating commercial plant by 2040. The panel will also advise DOE to participate in the \$1,000 million second stage of the International Thermonuclear Experimental Reactor (ITER), a long-term tokamak experiment that the United States, the Soviet Union, the EC and Japan are now planning, and request funds next year to begin construction of the US Compact Ignition Tokamak (CIT) at Princeton Plasma Physics Laboratory. If approved, CIT could be finished five years before ITER and would provide a solid physics foundation for the international project.

Next week, the US National Academy of Sciences is expected to recommend that the \$170-million-per-year US laser fusion programme should concentrate on upgrading existing facilities before starting a major demonstration facility. The report is likely to endorse spending \$300 million on the NOVA laser at the Lawrence Livermore National Laboratory, in order to raise its energy from about 100 kilojoules to 1 megajoule. That should be enough to reach break-even, although it will still provide only a tenth of the power needed for energy production. Plans for a reactor — known as the Laboratory Microfusion Facility — that could demonstrate energy-production techniques should be scrapped until early in the next century, the panel will conclude.

An independent panel of prominent European scientists and industrialists, chaired by Professor Umberto Colombo, from ENEA (the Italian energy research organization) has recommended that the EC extend the life of the current Joint European Torus (JET) reactor from 1992 until 1996. At its peak performance, JET is less than a factor of two away from break-even. But this can be maintained

only for seconds, after which impurities from the reactor walls begin to contaminate the plasma. Keeping JET running until 1996 could solve the problem, says Charles Maisonnier, director of the EC fusion programme. Ministers from EC states are not expected to reach a decision on JET's future before early next year, but Maisonnier has "strong hopes" that the extended project will win approval.

The EC report also says that Europe should retain the capacity to go ahead with a solely European proposal, the Next European Torus (NET), in case there are problems with the international collaboration. NET would cost the EC about \$3,900 million, compared with a share of the estimated \$4,900 million cost of ITER. Despite the uncertainties, the report recommends that the EC participate in the second phase of ITER planning as the 'next step' in the EC's fusion programme. ITER would serve as an experimental intermediate between JET and a demon-

UK NUCLEAR POWER

Third Hinkley reactor in limbo

London

UK ENERGY Secretary John Wakeham has given planning permission for a pressurized water reactor (PWR) at Hinkley Point in Somerset. But Nuclear Electric, the new state-owned generating company, must wait until 1994 before the government decides whether to pay for the plant.

The proposed Hinkley C PWR was the subject of a long-running public inquiry, which closed last December. Wakeham has accepted the recommendation of the inquiry chairman Michael Barnes, to grant consent for the plant, outlined in a report released last week.

Barnes concluded that the PWR's benefits — increasing the diversity of Britain's electricity supply while cutting carbon dioxide and sulphur dioxide emissions — will "substantially outweigh" local environment and health risks, and high cost estimates for PWR-generated electricity.

Spiralling estimates of the cost of nuclear electricity forced the Department of Energy to remove nuclear stations from the plan to sell the electricity industry into the private sector, and to put the expansion of the nuclear programme on hold pending a review in 1994. The cost of nuclear power, relative to other sources, will be an important factor in the decision on Hinkley C. Despite the uncertainties, Nuclear Electric chairman John Collier welcomed last week's decision, saying: "Our job is to make sure the 1994 review has a favourable outcome".

Peter Aldhous

stration electricity-generating reactor.

The first three-year conceptual design phase of the ITER programme has been based at the Max Plank Institute in Garching, West Germany, and is now nearing completion. All the international partners are expected to make a bid to host the second phase in their own geographic areas, on the assumption that it will give them an advantage when the time comes to pick a site for the facility itself.

But ITER's future remains uncertain: the thorny issue of intellectual property rights has not been fully worked out, and there is concern that the crumbling Soviet economy will not be able to meet its share of the costs. The UK Department of Energy is deferring its decision until the Colombo report has been studied in detail. Similarly, a decision by the US Department of Energy has been in abeyance, awaiting the report by the Stever committee. Even though ITER's second phase involves no construction, members will be expected to contribute as much as \$50 million a year to its design.

Christopher Anderson & Peter Aldhous
BROADCASTING

Satellite television faces a break

Tokyo

THE two million or so Japanese who have bought expensive antennas and tuners to receive satellite television broadcasts are left this week wondering if they will soon have only blank screens to watch. Japan's only broadcasting satellite, Yuri-2b (Lily-2b), will run out of the fuel necessary to maintain geostationary orbit in a few months and a replacement satellite, Yuri-3a, is seriously short of power.

Yuri-3a was launched successfully last week by the National Space Development Agency (NASDA), but is limping along on only 75 per cent of expected power because of a defect in the distribution circuit connected to the satellite's two solar panels. As a result, full television transmissions can be maintained for only two years "at best" instead of the seven years originally planned, according to Katsuo Yonezawa of NASDA's international affairs division. After that, one of the satellite's three transponders will have to be shut off and the national broadcasting corporation will lose one of its two channels. The second transponder will feed a new commercial channel.

The satellite failure comes on top of the loss of NHK's second broadcasting satellite, BS-2X, which was destroyed when a European Ariane rocket exploded. Yuri-3a will now be alone until next April at the earliest when NHK hopes to launch satellite BS-3H aboard a US Titan rocket to replace the lost BS-2X. **David Swinbanks**

Moths provide a model

London

THE UK Department of the Environment (DoE) is to fund a three-year £390,000 project to monitor the spread of a small Mediterranean moth now invading southern England, in the hope that the data will help to predict what would happen if genetically engineered insects were to be released in the future.

The firethorn leaf-miner moth *Phyllonorycter leucographella* was first noticed in Essex last year, and is still restricted to that county. Its caterpillars feed exclusively on firethorn *Pyracantha* spp., a common garden shrub. Professor John Lawton, from Imperial College's population biology centre in Ascot, who is leading the research, says that the moth may have been present in the United Kingdom for only two or three years, and may have been introduced with imported firethorn plants.

The invasion provides an ideal opportunity to study the spread of an insect population, according to Lawton, and will provide information that may influence DoE policy on the monitoring of releases of genetically engineered insects. The DoE believes that adequate monitoring of the release of genetically engin-

needs models that can predict the spread of released insects in a real "grainy" environment, Lawton says. As yet, there is no detailed information on the spread of any insect in the United Kingdom from which to develop these models. Lawton concedes that the firethorn leaf-miner's population biology may not be a typical example, but he points out that its association with a particular species of plant is useful. Genetically engineered insects may, in future, be used as biological control agents, attacking specific plant or animal pest species.

Commercial releases of genetically engineered insects for biological pest control are several years away, but small experimental releases to address purely academic questions may be a more immediate prospect. One application to

release has already been submitted, but was withdrawn before a decision was made, after the UK research councils and British Petroleum declined to fund it.

Lawton is concerned about the prospect of future commercial releases of genetically engineered insects. Possible biological control applications include the production of engineered ladybirds with increased voracity for their insect prey. These genes could enter the wild population, Lawton says, "with quite unforeseen consequences".

Professor David Bishop, from the Institute of Virology in Oxford, a pioneer in the development of genetically engineered virus insecticides, says that more ground work is needed to assess the environmental impact of commercial insect releases. But he is concerned that commercial companies may not wait for these data before applying for permission to release.

Peter Aldhous

ECOLOGICAL RESEARCH

Green science thinks bigger

Washington

ECOLOGISTS may not spend much time pacing the halls of Congress, but they seem to recognize a political opportunity when they see one. Faced with unprecedented interest in environmental research and congressional fondness for targeted science programmes, US ecologists are preparing their first shot at the big bucks. Early next year the Ecological Society of America (ESA) will release a broad research strategy for the 1990s and will also seek several millions of new dollars to finance it.

"For our society this is a landmark document — and a very risky one", says ESA president-elect H. Ronald Pulliam. Known as the "Sustainable Biosphere Initiative", the draft plan embraces much present ecology research, which will be repackaged to highlight its application to global environment issues.

But planners concede that they may risk angering some ecologists by their intention to assign priorities to their goals. Congress and the federal funding agencies are not stupid, they argue, and too broad an initiative dressing all continuing research in a fashionable green would fool nobody. Some current research must of necessity be left by the wayside.

So far, the gamble seems to have paid off. At a meeting of the Ecological Society of America in July, the proposal won solid endorsement. First responses to a 50-page draft sent to 500 scientists for comment suggest that "we're on the right track", according to Jane Lubchenco, the Oregon State University zoologist heading the drafting committee. The committee plans to have the document ready for a congressional briefing early next

year, with final publication in April.

The initiative is aimed at two environmental issues that need no selling — global change and biodiversity — and a third, called "sustainable systems", that may. Low-input agriculture is the common example of a sustainable system: with careful intermingling of complementary species, farm land can be made productive with minimal use of pesticides, fertilizers and chemicals. Ecologists may see the point, but can farmers be convinced to lend their substantial weight to this part of the initiative? Experience in agricultural biotechnology suggests that that will not be easy.

The initiative goes beyond research to emphasize public education and the application of ecology to public policy, which will entail convincing legislators and the public that ecology offers a cheap and intelligent way to solve environmental problems. ESA will then seek federal endorsement of the initiative, either by the Congress or the White House.

New money is, of course, likely to be a stumbling block, but ESA planners hope their document will extract funds from half a dozen federal agencies that already support environmental work.

There are some encouraging signs. A recent National Science Board report recommended \$20–\$50 million in new support for biodiversity research. Large ecological projects costing as much as \$200 million have been advocated as part of the study of climate change. And earlier this year, the National Academy of Sciences recommended another \$270 million over ten years for more forestry research, motivated partly by global warming concerns.

Christopher Anderson



ered insects will be possible, but Lawton suspects that the manpower required may be more than DoE can afford.

In order to see what level of monitoring is needed, Lawton will compare surveys conducted by his own small research team with records from "an army of volunteers", recruited by means of leaflets aimed at local natural history societies, conservation groups and sixth-form biology students. "We may get a nasty shock", he says, as monitoring by a small group of scientists may give a badly distorted picture.

The data collected may also allow biologists to develop better models of population growth. Most existing models examine a population spreading through a homogeneous environment, but the DoE

Due process protection

SIR—In an article headed "Investigators investigated" (*Nature* 346, 9; 1990), Christopher Anderson attributed to one of us, Suzanne Hadley, the assertion that "full due process" [the US term for such rights as appeal, hearing and cross-examination in a legal proceeding] is "inappropriate for OSI" (Office of Scientific Integrity).

We wish to make it clear that due process protection is reflected in all aspects of OSI procedures from start to finish. The due process protection in an investigation differs from that obtaining in a court of law or a hearing before an administrative law judge, but concern for due process is always appropriate for the OSI.

Specifically, the respondent in an OSI investigation receives formal notice of the investigation, together with a clear and complete statement of the issues that are the focus of the investigation. Respondents may be represented by counsel, and may provide any evidence and information they believe is relevant. They are presented with the evidence developed by the OSI and given an opportunity to respond, both in interviews and in written submissions. All interviews are recorded and transcribed, and the person interviewed has an opportunity to review, correct and comment on the transcript.

At the conclusion of an OSI investigation, the respondent (as well as the informant) is given the opportunity to review and rebut or comment on the draft report. Comments are appended directly to the report, which is revised and/or expanded, as appropriate. If misconduct is found, and sanctions proposed, the respondent has a further opportunity to review and comment on them.

Beyond the OSI, a careful process of review provides ample checks and balances on OSI investigations. Findings and recommendations together with the respondent's comments or rebuttal are reviewed by the relevant agency director and by the Office of Scientific Integrity Review. The Assistant Secretary for Health also reviews the findings and recommendations, before making the final decision. If debarment from receiving federal grant and contract funds is recommended, an opportunity for a new hearing is provided.

JULES V. HALLUM
(Director)

SUZANNE W. HADLEY
(Deputy Director)

Office of Scientific Integrity,
National Institutes of Health,
Building 31, Room B1C39,
Bethesda,
Maryland 20892, USA

Grant-making

SIR—With reference to your leading article ("Untransparent grants", *Nature* 346, 684; 1990), it is difficult to see how we in the Medical Research Council could be more transparent about our decision-making processes without engaging in a public discussion of the merits and demerits of individual research proposals. We do not consider that this would be in the best interests of the research community.

It should not be surprising that there is not total unanimity of view at the various stages of the peer review process — referees, visiting subcommittees and research board. Indeed, if there were, the procedure could be greatly simplified. At each stage, a wider scientific perspective is introduced. Research boards have to take account of how the quality of work in one research field compares with that of another. What is excellent in one field may not be so excellent when judged against competing claims from other fields and so the council is necessarily having to decline some applications that receive glowing testimonials from referees. These are the very difficult judgements that the peer review system is constantly having to make. They are judgements that have to be made regardless of wider considerations of scientific strategy, which are themselves made public in our corporate strategy document.

D. A. REES
(Secretary)

Medical Research Council,
20 Park Crescent,
London W1N 4AL, UK

Tenure in Japan

SIR—There is a curious omission in Robert J. Geller's otherwise quite sensible argument about the limited tenure positions available to foreign staff members in Japanese universities (*Nature* 345, 380; 1990).

Tenured academic appointments in Japanese universities presuppose proficiency in written and spoken Japanese, in which all formal administrative procedures are carried out, including faculty and committee meetings as well as work in labour unions and professional associations. Exemption from these duties for a foreign faculty member because of linguistic limitation will certainly invite complaints from Japanese faculty members, if only because of the unequal amounts of time to be sacrificed to activities other than creative scientific work.

The participation of more non-Japanese faculty staff in the running of Japanese universities would be welcome. But to expect bilingualism in Japanese and English in the administrative process is ludicrously utopian in the current ambience. Can anyone with experience in

Japanese universities suggest a possible way out of this perennial dilemma? Or should we expect there to be a cohort of competent scientists in the world with sufficient proficiency in Japanese to enable them to start with half the burden of administrative work, that, apart from the teaching job, the average Japanese academic must expect to shoulder?

ATUHIRO SIBATANI

Faculty of Humanities,
Kyoto Seika University,
Iwakura, Sakyo-ku, Kyoto 606, Japan

Clinicians wanted

SIR—Those responsible for preclinical education generally strive to expose medical students both to the newest developments in science and to the application of established science to the understanding of disease and its treatment. The effective teaching of clinical and applied science depends particularly on members of staff with a medical qualification. In contrast to those teaching much of biophysical, chemical and molecular science, medical graduates are recruited in competition with hospitals and other such institutions, which in turn compete with private clinical organizations. The young medical graduate contemplating a career in medical science usually has to accept a substantial reduction of career income.

John P. Gibson (*Nature* 346, 213; 1990), who asks what possible purpose can be served by separate salary scales for medical and science graduates, thus answers himself correctly when he observes that the purpose is recruitment. The principles are no different from those he can see in North America. In New Zealand, current university scales have all been negotiated in a common procedure; major considerations have been the problems of recruitment and retention of staff. The irritation produced by the formal distinction of the two different salary scales has to be contrasted with the academic disaster that followed adoption of a single scale some years ago. The alternatives practised elsewhere are not attractive: to maintain secret ways of meeting the market value of particular staff or, less palatable still, overtly or covertly to restrict preclinical teaching posts to medical graduates and thus to restrict the view of medical science to which future doctors are to be exposed.

Your correspondent is wrong in one major conclusion — our difficulty is not to attract first class scientists but to find graduates who can teach medical science from the secure base of personal experience in both preclinical and clinical elements of the curriculum faced by their students.

J. D. SINCLAIR

Department of Physiology,
University of Auckland, New Zealand

Disputes over monoclonal antibodies

Howard E. Greene Jr and Bradford J. Duft

Will increasingly bitter patent disputes stifle scientific medical research? A recent Commentary article about a sandwich immunoassay has provoked this exchange.

IN his Commentary article¹ "A shadow over immunoassay", Roger Ekins bitterly attacked the patent of Hybritech Inc. on a sandwich immunoassay. He claimed that the technique was "obvious", and therefore not patentable, and called into question the ability of the US patent system "to discriminate — in areas of increasing scientific complexity — between spurious claims to novelty and genuine invention". Ekins also attacked Hybritech and its employees in what we believe to be an unsupportable manner. He was an interested party, both in being an immunochemist and in acting as an expert witness for Abbott in its legal contest with Hybritech. Ekins' article, in essence, summarized his testimony that had been rejected by the courts. We too are interested parties — one a co-inventor of the assay and the other a co-counsel for Hybritech during litigation over the patent — and we wish to put the record straight.

Ekins' article confirms that, in the 1970s, he was truly an expert in the field. Yet he misunderstands the objectives of the US patent laws and the analyses conducted under them for determining patentability. Ekins shows that he is aware that the law precludes issuance of a patent if the subject matter would have been 'obvious', but makes no mention of the limitations imposed by law on the determination of 'obviousness'. He does not focus on what must have been obvious, to whom it must have been obvious and when it must have been obvious.

Nonobviousness

Under US law, what must have been obvious is the invention as a whole. In the case of Hybritech's patent, the invention is not monoclonal antibodies generally, the use of monoclonal antibodies in immunoassay formats generally, or even the use of monoclonal antibodies in all sandwich assays. The invention is, instead, only the use of particular types of monoclonal antibodies in certain forms of sandwich assay. Accordingly, Ekins' view that the use of monoclonal antibodies in immunoassays generally may have been a topic of international contemplation, even if true, misses the point.

US patent law also specifies the time of the obviousness inquiry to be that time "when the invention was made". Hybritech introduced evidence in the courts showing the conception of the invention in January 1979. It is not clear

from Ekins' Commentary just how much of the evidence he is relying on to establish obviousness stems from before that date. Our experience is that much of the scientific work cited by others on this question was done well after January 1979.

Finally, and perhaps most important, US patent law specifies that to deny a patent, the invention must have been obvious to people of "ordinary skill" in the "art to which the invention pertains". It is difficult to determine what would have been obvious to such a person in 1979 from opinions expressed with the benefit of hindsight in 1989. For example, Ekins claims that "I submitted a grant application (approved in 1977) for the development of monoclonal-antibody-based sandwich and competitive hormone assays for use in the WHO human reproduction programme". But because of the vagaries of memory and perception — and bias — US courts grappling with the question of obviousness often look to objective indications in reaching the legal conclusion. Such objective evidence includes failure of others, evidence that workers in the field were deterred from developing the invention, and commercial success of the invention once introduced. The evidence produced before the courts showed unambiguously that others had failed to develop assays of the sort claimed by Hybritech.

There was also substantial evidence that those working in the 'trenches' of commercial immunoassay design were deterred by worries that monoclonal antibodies might not exhibit the affinities required for successful immunoassays and that the narrow specificity of monoclonal antibodies might make them unsuitable for commercial use². Finally, the particular sandwich assays covered by the Hybritech patent have been an immense commercial success, both in Hybritech's hands and in the hands of infringers. Not only does such widespread adoption of a new invention support a conclusion of 'nonobviousness', it also answers Ekins' assertion that there is no evidence of the superiority of the patented technology. If it were not better, people would not use it — and infringers would neither take it nor fight for it.

The case put forward by Ekins has been twice considered and then rejected by US courts. After a first trial in a district court³, the Court of Appeals for the Federal Circuit (which has special patent expertise and is the court where all US patent appeals are heard) rejected these same

arguments against Hybritech's patent, and the Supreme Court (the highest in the land) let the decision stand⁴. Another district court in Los Angeles subsequently rejected the arguments⁵, and the same Court of Appeals affirmed that decision⁶.

The first opinion by a unanimous Court of Appeals was written by Judge Giles S. Rich, one of the authors of the US patent statutes and one of the most respected and well-known patent jurists in the world. His judgement on all the facts in view of the law is in our opinion of much more value than Ekins' verdict on his 'facts' alone.

Judgement

The Court's judgement is supported by the actions of Hybritech's competitors. Monoclonal Antibodies Inc. and Abbott Laboratories. These defendants took the Hybritech invention because it is better than anything that went before. Ekins asserts that Hybritech did not "produce any data of the kind normally required in this field demonstrating their successful development of an accurate, reliable immunoassay using such antibodies". But Hybritech developed accurate, reliable immunoassays using monoclonal antibodies according to its patent many times over. So did Abbott and Monoclonal, the infringers. One can only conclude that, were this not so, Abbott and Monoclonal would not have used the Hybritech invention instead of their own technology.

Every ground that might be argued for the invalidity of a US patent was first raised against Hybritech by lawyers hired by Monoclonal to defend it. All these arguments were rejected without reservation after consideration of all evidence mustered by both sides. Subsequently Abbott, notwithstanding all the money and lawyers it was able to throw at Hybritech, suffered the same fate.

But perhaps the simplest rebuttal to Ekins is to note that Abbott has now effectively endorsed a view opposite to him. It has settled its dispute, plainly admitting the validity of Hybritech's patent. Ekins hedges against Abbott's settlement by stating that "costly legal disputes between large companies are, in practice, often resolved by private agreements between them, thus retaining important patents in place to their mutual benefit". The implication seems to be one of collusion between the settling parties to impede access to the subject technology. Yet such agreements invariably reflect economic

decisions arrived at when the perceived returned on investment from further litigation drops below the threshold required to justify continuing. Given Abbott's huge commercial stake in the immunoassay market relative to the legal fees of patent litigation — and their losses in court — the company almost certainly settled because it believed it had no case and could not win. No more, and no less.

We do not understand the seeming disdain for patents reflected in Ekins' article. There is no need to detail here the benefits of patents; it is enough to mention the existence of a patent system in every country of note in the world.

Two hundred years ago, to the year, the United States passed its first patent statute as one of the country's first acts after ratification of the constitution in 1789. The constitution had given Congress the power "To promote the progress of . . . useful arts, by securing for limited times to . . . inventors the exclusive right to their . . . discoveries"⁷. Writing later⁸, James Madison stated that "The utility of this power will scarcely be questioned".

Indeed, the implementation of this power through creation of the US patent system contributed in no small measure to the prominence of American invention and of the nation. At about the turn of the century, a Japanese official was sent to the United States to analyse its meteoric rise. He reported that "We have looked about to see what nations are the greatest, so that we can be like them. We asked ourselves 'What is it that makes the United States such a great nation?' and we investigated and found that it was patents, and we will have patents" (ref. 9).

Another aspect of the value of the patent system was summarized by Dews¹⁰:

Except for the patent right, men of limited means would be hopeless in negotiating with the large corporation. Without the patent right, if he did not have sufficient funds to market the invention himself, he would be compelled to submit his invention to financiers. If these financiers should decline to make a fair deal with the inventor and the inventor refused to accept a deal considered unfair, they would ignore him and proceed to market the invention without his consent and he would be helpless to stop them.

In this case, Abbott, a giant pharmaceutical company, was alleged to have made a sustained effort not only to pirate tiny Hybritech's invention but to destroy the company and plunder the market it created. Hybritech's patent turned out to have "a protective character like that of a high powered rifle in the hands of a puny man beset by a wildly charging bull elephant" (ref. 11).

In any event, Ekins' fear has been realized: maintenance of Hybritech's patent. We do not share his concern of adverse consequences for scientific progress, in part because patents are, by

law and in fact, primarily relevant only in the commercial arena. It is plain that progress in molecular biology and genetic engineering has not been slowed by Stanford University's Cohen and Boyer patent. Similarly, scientific progress with monoclonal antibodies has in no way been affected by Hybritech's patent on the sandwich immunoassay, and Ekins has not identified any such difficulties in any setting — commercial or academic.

For 200 years, US patent law has served the best interests of society. We submit that it will continue to do so, not least in immunochemistry and all areas of science.

Howard E. Greene Jr and Bradford J. Duft are at Amylin Corporation, 9373 Towne Centre Drive, Suite 250, San Diego, California 92121, USA.

EKINS REPLIES — My Commentary article was written long before I learned of the Hybritech/Abbott agreement, and I wrote as an independent academic scientist, not as an "allied witness" of the Abbott company. I have no antipathy to Hybritech *per se*, and would willingly give evidence on Hybritech's behalf if it were threatened by another company on grounds that I believed scientifically wrong.

The immediate issue is whether the use of monoclonal antibodies for immunoassay purposes in general, or any kind of immunoassay procedure in particular, was or was not "obvious". In my own opinion it was totally obvious, since there was no known reason why such antibodies should not be used as a replacement for those produced by conventional methods. This is not a conclusion reached with the benefit of hindsight: the potential advantages of synthesizing antibodies using Milstein and Köhler's new methodology was repeatedly discussed long before the "conception date" claimed by Hybritech (although it was inevitable — for numerous practical and financial reasons — that this obvious development could not be immediately implemented, and that identifying antibodies suitable for particular analytical applications would take time). Meanwhile, despite Greene and Duft's argument, Hybritech's clear intention was to patent the use of monoclonal antibodies in as many immunoassay formats as examiners would allow, as evidenced by their other patents^{12,13} covering such use in "inhibition assays". Many disinterested researchers nevertheless agree that the true invention was Milstein and Köhler's novel method of antibody synthesis, whatever legal sophistries have subsequently been used to disguise this simple fact.

The wider issue raised by this case is whether existing legal institutions are capable of identifying genuine inventions. My own experience in this area, admittedly limited, has led me to believe that they are not. I have, for example, noted that patent

offices are unduly concerned with words *per se* rather than with the concepts they represent. In a recent case, a patent office has agreed to the granting of a patent for a new immunoassay methodology provided it is restricted to the measurement of hormones, as a hormone was used to exemplify the method's use in the patent application. But the biological function of the substance cited was entirely irrelevant, the methodology described in the patent clearly being applicable to any substance (that is, an antigen) capable of reaction with an antibody.

In the present case, the courts appear to regard a 'monoclonal antibody' as different, in its antigen-binding properties, from one produced by normal methods. Had the term 'mono-site-specific' antibody been used in the patent application, I suspect the lack of novelty would have been perceived immediately, as such antibodies can be prepared by conventional means. Similarly, my experience of patent litigation suggests that the courts can arrive at nonsensical conclusions, partly because the judges concerned — though perhaps excellent lawyers — are unfamiliar with the scientific issues at stake, and partly because normal court procedures may prevent the proper expression of informed scientific opinion on key points of contention.

Because academic science is increasingly affected by the burgeoning tendency of universities and companies to patent every idea that has the slightest hope of being presented or misrepresented as a genuine invention, it is legitimate to question whether major decisions in this area can be left to individual companies, aided by lawyers with limited scientific knowledge, in far-off courts of law to which academic researchers have no access, and in which they have no voice. This is the fundamental question raised by the Hybritech patent, and one which it is right and proper to debate in a scientific journal. □

Roger Ekins is in the Department of Molecular Endocrinology, University College and Middlesex School of Medicine, London W1N 8AA, UK.

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5. *Hybritech Incorporated v. Abbott Laboratories*, 4 U.S.P.Q. 2d 1001 (C. D. Cal. 1987).
6. *Hybritech Incorporated v. Abbott Laboratories*, 849 F. 2d 1446, 7 U.S.P.Q. 2d 1191 (Fed. Cir. 1988).
7. Article 1, Section 8, Clause 8.
8. *The Federalist* No. 43, at 267.
9. *Cong. Rec.* at 901 (20 January 1962).
10. Dews, G. *The Patent Right in the National Economy of the United States* at 140 (1952).
11. *Coe. TNEC Hearings* Part III, at 856 (1939).
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13. UK patent application GB2086041A. *Immunoassay Utilising Monoclonal Antibodies*.

Time to make friends of chemists

The contemporary chemistry journals deserve to be more widely read, not just for what chemists have to say about the natural world, but for the daring of the empirical generalizations in which chemists seem to specialize.

CHEMISTS are much misunderstood, at least in part because of their publishing practices. Potential well-wishers have been kept from recognizing the value of what they do by the way chemistry journals are spattered with articles described as "Part XIII" in a continuing study of some class of compounds, texts are filled with formal characterizations of whichever compounds may have been synthesized in the course of a study, much as taxonomists record the classification of new species.

Yet ask a chemist what he or she is about, and you will be given a different tale. First, of course, you will be told about the useful arts, the processes for making materials as different as polypropylene and Semtex (the 'plastic' explosive used for blowing up civilian aircraft). At least to outsiders, there now seem no limits to what people can synthesize. They are even prepared to do it with one hand tied behind their back: a recent issue of *Journal of the American Chemical Society* contains an account by Nakatsuka *et al.* (112, 5583; 1990) of a synthesis of the immunosuppressant FK506 in which two specific carbon atoms were substituted by ^{13}C , and in which the authors used α -bromoacetic acid in their synthesis scheme because it is readily (and cheaply) available as a material in which both carbons are enriched in the heavy isotope.

Yet chemists' most engaging habit is their confidence that chemistry is an explanation of virtually everything. Physicists may break their heads over the existence of the top quark, or whether axions can supply the missing mass of the Universe, but chemists will not shrink from explaining why Teflon has virtually no friction or why zeolites function well as catalysts for cracking hydrocarbons.

Outsiders should not disparage such impatient ambition. Chemists are masters at elevating empirical generalizations into natural laws. Is there not, for example, Trouton's rule that the latent heat of vaporization of a liquid in calories is roughly 22 times the boiling point (in degrees kelvin)?

For half a century after Mendeleev, the periodic table of the elements must have seemed to most of them to be a preferred substitute for Aristotle's elementary bundle of earth, air, fire and water. The arrival of the quantum theory and, in particular, of Pauli's exclusion principle (which explains the periodic table except for the three-quarters of the elements

whose atomic number is greater than that of iron) did not seem to chemists of the time to be an undercutting of their theory, but rather a confirmation of what they had been saying all along. If only somebody, in 1917, had had the wit to announce that Einstein's general theory had confirmed that newtonian mechanics is essentially correct, physicists would have avoided much subsequent trouble.

Generalizations based on observations have at least the virtue that they are generalizations of the kind ordinary people reach in ordinary life. Could that have been, in past decades, one of the reasons why chemists were able to attract large numbers of others like themselves to follow their own pursuits? More recently, faced with falling enrolments in student courses, chemists and their professional organizations have been tempted to emphasize the useful arts. Might they not find it at least as effective to describe chemistry as a way of reaching accessible, if sometimes empirical, explanations of the natural world?

Here is another illustration, from the same issue of *J. Am. chem. Soc.* as the synthesis of FK506 and the account (see last week's *Nature* 347, 13; 1990) by Hunter and Sanders of how the interaction between conjugated electron systems can be simplified.

Take, for example, pyridine, the analogue of the conjugated six-carbon benzene molecule in which one of the carbon atoms is replaced by a nitrogen, with the result that the molecule contains only five hydrogen atoms (one for each carbon). Should this not be essentially the same as benzene? Not exactly. Pyridine resembles benzene in the old Kekulé picture of conjugated systems in which the successive atoms in the six-membered ring are joined by alternating single and double bonds, but then there are two electrons left over (nitrogen has five electrons in the second shell, but only three are used up in making bonds), usually termed a 'lone pair'. Hand-waving over many years has been taken to suggest that this is why pyridine is more reactive chemically than benzene. More to the point, calculations show that the ground-state of the molecule is indeed planar, like that of benzene.

But what about the excited states of pyridine? The analogy with benzene is no longer reliable, for the two spare electrons on the nitrogen atom are natural candidates for excitation. Apparently the spectroscopy of pyridine has been a head-

ache, sorted out only in the past few years by the spectral observation of deuterated pyridine in crystals of deuterated benzene. The first excited state is a triplet state, and the observations show the molecule to be boat-shaped (with the nitrogen atom lifted from the plane by about 35° , the diametrically opposite carbon atom by about a third as much in the same direction).

Why should this be? W. J. Buma, E. J. J. Groenen and M. C. van Hemert from the University of Leiden have calculated the most likely configuration and have concluded that a boat-shaped geometry of the excited state is indeed the most stable (*J. Am. chem. Soc.* 112, 5447; 1990). There are many who would have put out flags at that stage, and turned their attention to the next problem on their agenda. But chemists are different. They want a picture of what has happened, and what may happen with other similar molecules.

So this interesting article concludes with an interpretation of what the Cray machine has said in almost anthropomorphic language. With seven electrons (one excited) in a planar conjugated system, the nitrogen atom would no longer be firmly bound to its adjacent carbons, with the consequence that the electron states on those two carbons "rehybridize themselves" so as to form bonds with the nitrogen atom more nearly like those in aliphatic carbon compounds. There follows entirely plausible brooding about the circumstances in which other molecules may behave similarly. Another rule of thumb seems to be in the making.

There is, of course, nothing wrong with such a way of doing business. Nobody plans to erect a generalization on the basis of a single calculation which, in the case of pyridine, has provided a neat confirmation of an experimental result and which only fortuitously provides grist to the mill, familiar enough to chemists, of rehybridization (which is but a name for the grouping of valence electrons on single atoms into new, but equivalent, linear combinations). But people with a more purist caste of mind would instead be worrying about questions such as whether the Born-Oppenheimer approximation that usually justifies the supposition that nuclei are immovable on the timescale of electron calculations can be valid for the excited state of pyridine. Perhaps it is not surprising that chemists lose patience with them.

John Maddox

High H_0 ?

M. Fukugita and C. Hogan

EVER since Hubble discovered the linear relationship between the velocity v of recession of a galaxy and its distance r — the relation $v = H_0 r$ which shows that the Universe is expanding — astronomers have attempted to establish the value of the constant H_0 that bears his name. The value not only determines the size scale of the Universe but also its expansion rate, so that our entire theoretical framework for the global structure of the Universe and its history revolves around it. The difficulty has always been to find what the distance r is, especially to galaxies sufficiently far away for local 'peculiar' velocities, as deviations from a pure Hubble expansion are called, to be negligible. Countless studies have left the value of H_0 still uncertain by about a factor of two. Now, in the most recent¹ of a series of five papers, Jacoby, Ciardullo and Ford have presented a new and precise measurement of the distance to the Virgo cluster, a key link in the chain of measurements to distant galaxies. Their precise measurement of the distance to crucial mid-range galaxies in the Virgo cluster allows an evaluation of H_0 to within 15 per cent. To the consternation of some cosmologists, the actual value seems to be at the high end of the scale, leaving serious difficulties for the standard theories.

Astronomical candles

Measuring a distance ratio requires a standard candle — a recognizable thing whose true brightness is always the same. The distance ratio of two galaxies can be inferred from the ratio of the brightness of the standard candles in the galaxies. No astronomical candles are exactly standard, but the two best extragalactic candles — Cepheids, a kind of bright variable star, and Type Ia supernovae, a kind of exploding star, allow relative distances between individual galaxies to be measured to a fractional accuracy of about a tenth^{2,3}. To measure an absolute distance, one must establish a chain of reasoning (sometimes called a distance ladder) using a sequence of distance ratios, ultimately tied to some known distance. Thus the absolute extragalactic distance scale is ultimately tied to a distance on the Earth.

The problem for years has been that the Cepheids are observable only in nearby galaxies, whereas the supernovae are so rare they are typically only found far away (the recent nearby supernova, SN1987A, being the wrong kind). In particular, Cepheids have not been observable in the nearest large galaxy cluster, the Virgo cluster, where they could be used to calibrate the supernovae and test other

candles all at a single distance; this is why spotting the Virgo Cepheids is a top priority for the Hubble Space Telescope.

The link from the nearby galaxies to the Virgo cluster and beyond has thus mostly rested on other techniques, such as the Tully–Fisher relation^{4,5}, which use properties of whole galaxies as standards. Such methods have been treated with caution because they have potential flaws, such as the small numbers of local calibrating galaxies, and the fact that the best indicators (spiral galaxies) tend not to lie in clusters. Most importantly, because they are not as accurate as the Cepheid and supernova candles, statistical samples must be introduced whose membership always leads to some ambiguity, potentially even to a biased result. Moreover, unlike Cepheids and supernovae, it is not understood why these techniques should work as well as they appear to do, as the state of a complicated dynamical system like a galaxy depends on its detailed history, whereas the way a star evolves depends only on its mass and composition. Over the years most of these shortcomings have been overcome by careful cross-calibration, so that the main remaining weak point has been lack of confirmation by an entirely different method, preferably a fundamental standard. As it turns out, the accuracy of the cosmic distance scale based on the Tully–Fisher relation is fully confirmed by the new distance measurements.

Jacoby *et al.*¹ have introduced a new set of techniques which use bright planetary nebulae as standard candles. These nebulae are clouds of shining gas formed by stars near the end of their life, after they have exhausted their initial supply of hydrogen fuel and are burning helium. As a star gets older its core contracts and gets hotter, slowly at first, but eventually very rapidly. The rapid increase in energy from the core of the star hits the outer layers and blows them off. The most extreme cases are the explosive Type II supernovae of high-mass stars; but in stars of lower mass, things proceed at a gentler pace and the envelope floats away more slowly, forming a spherical shell of gas around the star. It seems these nebulae almost never get brighter than a certain limiting luminosity, which is what makes them good to use as standard candles. Jacoby *et al.* use the brightness distribution of a population of such nebulae; as a bonus, the data also test the theory of the evolution of stellar populations.

It is unclear why the upper limit of brightness is so precisely defined. But few of the events in the core of a star after hydrogen burns to helium depend much

on the initial composition of the star, or even, over a certain range, on its mass. For example, helium burning in low mass stars is initiated in a sudden flash during the catastrophic collapse of a 'degenerate' core of hydrogen; this collapse always occurs at the same universal core mass (the Chandrasekhar mass), which depends only on physical laws. So, it is at least plausible that the maximum mass of the helium in stars powering planetary nebulae should be standard.

These planetary nebulae have several other advantages as standard candles. For example, because they appear around old, low-mass stars, they are usually far away from the dusty stellar nurseries where brightness is affected by obscuration. This also means that they are found in the elliptical galaxies which most often populate galaxy clusters, as well as in the spiral galaxies calibrated by Tully–Fisher.

Spectral signature

The authors' technique exploits another crucial feature of these nebulae — as a result of fluorescence from the ultraviolet light coming from the hot central star, a significant portion of their luminous energy appears in very narrow spectral lines. This spectral signature means that they are easily recognized, and easily picked out even in distant crowded fields by taking images in narrow-band filters. Observations need only be taken at a single epoch, in contrast to the careful multiple-epoch photometry needed for both the Cepheid and supernova candles. Most significantly, planetary nebulae are bright enough in the line radiation to be measured accurately with current technology at the distance of the Virgo cluster. Besides giving a distance to Virgo, this meant that Jacoby *et al.* could choose six galaxies (within the cluster) known to lie at about the same distance but which differ as much as possible from each other in properties such as metallicity: in this way, they verified that the technique is not sensitive to such variations.

The errors are small enough to check the accuracy of the method against other methods. The ratio of the distance of two important nearby galaxies, M31 and M81, measured by planetary nebula distances agrees with the Cepheids. Three of the galaxies in Virgo have planetary-nebula distances as well as type Ia supernovae; both indicators agree that the galaxies are equidistant from us. The distance ratios of M31 to M81 and to Virgo measured with planetaries agree with the whole-galaxy Tully–Fisher method. Planetary nebulae can be used as a calibrator for the distances of elliptical galaxies in the Leo group, allowing the calibration of two other methods^{6,7} applicable to elliptical galaxies, and the distance ratio of the Leo galaxy group to Virgo measured with the nebulae agrees with these methods.

Thus, all the lowest-dispersion and best-tested indicators agree with each other where they overlap, and for each rung of the extragalactic distance ladder from M31 out to Virgo there are at least three independent, unrelated methods that give the same answer. Although there are certainly still some distance indicators that refuse to fall into line⁸, for the most part even their advocates voice doubts about their accuracy and reliability. The methods that agree with each other are also those that have had their internal consistency and their possible systematic flaws most thoroughly checked. The bottom line of the new paper is that the distance to the Virgo cluster — actually, an average of the distance to the six galaxies measured (the technique is accurate enough to measure the differences in their distances within the cluster itself) — is 14.7 ± 1.0 megaparsecs (Mpc).

Deriving H_0 from the Virgo distance can be done in several ways, but all of them yield a disconcertingly high Hubble constant, in the range $75\text{--}100 \text{ km s}^{-1} \text{ Mpc}^{-1}$. Such a rapid rate of expansion implies that the Universe is surprisingly young and small. In a universe without a cosmological constant (a kind of repulsive gravity) this rate is not consistent with conventional age estimates based on stellar evolution and nuclear-decay chronometers. The option of having a cosmological constant is anathema to theorists who both abhor fine tuning and free parameters in the grand design of the Universe, and like to believe in their chronometers.

With a high H_0 the density of ordinary baryonic (nuclear) matter allowed by the standard model of cosmic nucleosynthesis becomes much less than the dynamically measured density of dark matter, so that some exotic form of particle dark matter is required.

The short distance to Virgo would also require a downwards revision of the peak blue brightness of type Ia supernovae, implying that the theorists have somehow overestimated the total mass of radioactive nickel which powers the luminosity, or miscalculated the allocation of the energy budget between kinetic energy, blue light and other forms of radiation, by maybe 50 per cent. Perhaps theoretical inaccuracy at this level is not too difficult to imagine, as no nearby type Ia supernovae have appeared to test models in detail. A considerable amount of theoret-

ical tinkering was required to match the observations of SN1987A; perhaps some is now called for to match this new calibration of type Ia brightness.

For all of these reasons, Jacoby *et al.*'s conclusions will certainly still be greeted with scepticism, and can be expected to spark lively debate until proposed

repairs on the Hubble Space Telescope allow a test of their distance to Virgo with another, comparably accurate technique — the Cepheids. □

M. Fukugita and C. Hogan are at the Yukawa Institute for Theoretical Physics, Kyoto University, Kyoto 606, Japan.

PHARMACOLOGY

The dopamine connection

Solomon H. Snyder

THE revolution in psychiatry wrought by the introduction in 1952 of chlorpromazine as an antipsychotic agent led within a decade to dozens of successor drugs and abundant research to explain the varying therapeutic properties and side-effects of a wide range of drugs affecting the nervous system, called neuroleptics. The molecular cloning of a novel dopamine receptor by Sokoloff *et al.*¹ on page 146 of this issue is a significant step forward in this effort.

Initial notions that antiadrenergic effects explained the therapeutic effects of chlorpromazine never sat comfortably with the findings that more potent antiadrenergic agents lacked antipsychotic efficacy. In 1963, Carlsson and Lindqvist² observed that neuroleptic agents increase concentrations of dopamine metabolites in the brain and suggested that these alterations reflect increased neuronal firing, secondary to dopamine receptor blockade. Nine years later, Greengard and colleagues³ showed that dopamine can selectively enhance the activity of adenylyl cyclase in corpus striatum homogenates, with neuroleptic agents blocking the effects of dopamine.

However, butyrophenones, the most

potent neuroleptics, are notoriously weak in blocking dopamine, leading many investigators to conclude that dopamine plays no part in the action of neuroleptic drugs. The introduction of ligand-binding techniques to label dopamine receptors^{4,5} salvaged the neuroleptic-dopamine connection. Dopamine receptors labelled with [³H]dopamine resemble the dopamine-sensitive adenylyl cyclase with butyrophenone neuroleptics being weak antagonists. By contrast, dopamine receptors labelled with [³H]butyrophenones are influenced by butyrophenones as well as phenothiazine neuroleptics with potencies correlating very closely with clinical antipsychotic potencies^{4,7}. Sites associated with stimulation of adenylyl cyclase and labelled by [³H]agonists are now termed D₁ receptors, whereas those binding [³H]butyrophenones are the D₂ receptors, thought to be primarily responsible for the antipsychotic actions of all neuroleptic agents⁸.

Some recently developed drugs, usually termed 'atypical' neuroleptics, have a lesser incidence of extrapyramidal, parkinsonian-like side effects than classical neuroleptic drugs and may be more beneficial in treating the 'negative' symptoms

of schizophrenia, such as affective withdrawal. If the blockade of D₂ receptors is responsible both for therapeutic and extrapyramidal effects, then all neuroleptics should elicit the same incidence of extrapyramidal side-effects at therapeutically comparable doses. This discrepancy has been attributed to varying anticholinergic actions of neuroleptics as well as to the possibility that dopamine receptors in the corpus striatum, which regulates motor activity, differ from those in limbic, emotional parts of the brain, where antipsychotic effects are presumably mediated. But ligand-binding studies have not consistently revealed molecular differences in D₂ receptors in various brain regions. ►

DOPAMINE RECEPTOR SUBTYPES

	D ₁	D ₂ *	D ₃
Highest brain densities	Neostriatum (caudate)	Neostriatum (caudate)	Paleostriatum (Islands of Callejae, nucleus accumbens)
Posterior pituitary	No	Yes	No
Nigral dopamine cells	No	Yes	Yes
GTP Regulation	Yes	Yes	?
Adenylyl cyclase	Stimulates	Inhibits	No effect
Affinity for dopamine	Micromolar	High nanomolar	Low nanomolar
Butyrophenone potency	Micromolar	Subnanomolar	Nanomolar
Phenothiazine potency	Nanomolar	Nanomolar	Nanomolar

*Two D₂ receptor subtypes, designated A and B, arise from alternative splicing, but display identical drug specificities.

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The new dopamine receptor subtype D_3 , cloned by Sokoloff *et al.*¹ may clarify many of these dilemmas and provide powerful new strategies for developing safer, more effective antipsychotic agents. Sokoloff *et al.* screened cDNA and genomic libraries using probes based on the cloning of dopamine D_2 receptors by Civelli and associates⁹. The D_3 receptor displays the seven putative transmembrane-spanning domains characteristics of G-protein-linked receptors and about 50 per cent of its amino acids are the same as those of D_2 receptors. Nevertheless, the drug specificity of D_3 receptors expressed in transfected cells in culture differs in important ways from D_2 receptors, as does the localization of mRNA encoding the two receptor proteins in the brain (see table).

These differences may have substantial clinical relevance. For instance, the dopamine agonists pergolide and quinpirole have therapeutic effects in parkinsonism — actions previously assumed to reflect the stimulation of D_2 receptors. But because these drugs are between 30 and 100 times more potent when interacting with D_3 than D_2 receptors, it is more likely that their therapeutic activities are mediated through D_3 receptors. Their major side-effects are psychotomimetic actions, presumably mediated by dopamine receptors in the limbic regions where D_3 receptors are more concentrated than D_2 sites.

The different locations of D_2 and D_3 receptors may help explain the much greater incidence of extrapyramidal side-effects with butyrophenone neuroleptics such as haloperidol than with atypical neuroleptics such as clozapine and sulpiride. Haloperidol is about 20 times more potent at D_2 than D_3 receptors, whereas the atypical neuroleptics have fairly similar potencies at the two receptors.

Designing novel neuroleptics selective for D_3 receptors may diminish pituitary-related side-effects. By blocking D_2 receptors in the pituitary, classical neuroleptic agents increase the concentration of prolactin in the blood, causing amenorrhea, impotence, and, in some animal studies, an increased incidence of mammary carcinoma. Although there are very high densities of D_2 receptors in the lactotroph cells of the pituitary, they appear devoid of D_3 receptors. Of all the neuroleptics thus far evaluated, none displays more than a fourfold selectivity for D_3 over D_2 receptors. Medicinal chemists should be able to sculpt highly selective D_3 agents free from pituitary side-effects.

The identification of D_3 receptors may clarify some confusing aspects of dopamine research. For instance, ligand binding to the expressed D_3 receptors is not influenced by GTP derivatives, which regulate binding associated with D_2 and D_1 receptors. In the corpus striatum, ligand binding associated with dopamine

receptors on intrinsic neurons is regulated by GTP, whereas receptors on terminals of the corticostriate pathway are resistant to GTP^{10,11}: conceivably, the corticostriate dopamine receptors are of the D_3 subtype. Indeed, differentiating between the functions of the subdivisions within the corpus striatum may be a particularly notable consequence of the identification of the D_3 receptor.

The dorsal striatum, including the caudate nucleus, is comparatively enriched in D_3 receptors and contains terminals of the nigrostriatal dopamine pathway involved in Parkinson's disease and the extrapyramidal side effects of neuroleptic drugs. The more ventral corpus striatum, selectively enriched in D_2 receptors, includes the olfactory tubercle, islands of Callejae and nucleus accumbens, which are linked to phylogenetically older limbic areas of the prefrontal cerebral cortex, and are the likely sites for antipsychotic drug activity.

In recent years, progress in the search for safer and more effective antipsychotic drugs has been slow, with many workers turning away from dopamine receptors to focus on new sites such as sigma re-

ceptors¹². The discovery of D_3 receptors may rejuvenate research on dopamine, which in the past has given rise to the most important therapeutic agents used in psychiatry and neurology. □

Solomon H. Snyder is at the Johns Hopkins University School of Medicine, 725 North Wolfe Street, Baltimore, Maryland 21205, USA.

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BIOGEOCHEMICAL CYCLING

Bombs and ocean carbon cycles

J. R. Toggweiler

FOR geochemists, the radioactive fallout from the atom bomb tests in the 1950s and early 1960s was a scientific windfall. Studies that tracked the movement of the bomb isotopes out of the atmosphere and into the natural reservoirs of land and sea established an observational basis for the modern view of chemical transport. Thirty years later, the isotopes continue to reveal new facets of the natural world. On page 172 of this issue¹, Druffel and Williams use the penetration of bomb ¹⁴C into the organic carbon pools of the ocean to highlight the importance of a large, but poorly understood, reservoir of dissolved organic carbon in the ocean's carbon cycle.

The standard model for the ocean's carbon cycle is built around the gravitational settling of biogenic particles. The sinking particles take carbon from the upper ocean, where CO₂ is fixed by phytoplankton, into the deep sea, where the organic material in the particles is metabolically remineralized or buried in the sediments. The sinking flux, comprising macroscopic biogenic aggregations which include the remains of large phytoplankton, animal tests and animal egesta large enough to sink rapidly^{2,3} removes about 10 per cent of the carbon fixed by primary production in the euphotic upper layers of the ocean⁴.

The standard model has lately come under seige owing to evidence that the primary pathway for transporting organic material through the water column

involves dissolved, not particulate, organic compounds. This viewpoint is based on the recent discovery by Suzuki and Sugimura^{5–8} of a large pool of dissolved organic compounds in the upper ocean. The maximum concentration of Suzuki and Sugimura's dissolved organic carbon (DOC), around 300 µM, is found at the surface in the middle latitudes of the ocean. The concentration of DOC decreases with depth in parallel with the disappearance of oxygen, suggesting that the lion's share of the oxygen consumed in the ocean's interior is being used to oxidize dissolved organic matter, not particulate organic material. Analytical difficulties in reproducing Suzuki and Sugimura's results have made it difficult, however, to verify that this characterization of the ocean's carbon cycle is correct.

Druffel and Williams¹ now add new evidence that supports the dissolved organic pathway. They have measured the degree to which the ¹⁴C produced by nuclear weapons testing has contaminated the particulate organic carbon (POC) pools, both sinking and suspended, in the North Pacific. At present, inorganic CO₂ in the water below the upper kilometre of the ocean is uncontaminated with respect to bomb ¹⁴C. This is not true, however, with respect to the organic carbon in particles. Large settling particles can sink to the bottom in less than a year². Settling particles are also continually 'repack-

aged'. They break up and are re-formed, and in doing so exchange material with the pool of small particles suspended in the water column. The incorporation of suspended particles into sinking particles is such that the residence time of suspended particles in the deep sea is only 5–10 years⁹. According to the behaviour expected of particles, all the POC now in the ocean, 30 years after the bomb tests, should have $^{14}\text{C}/^{12}\text{C}$ ratios which are virtually identical to the bomb-contaminated levels observed at the ocean's surface. Druffel and Williams observe that suspended POC below the surface is significantly depleted in ^{14}C with respect to the upper ocean. They argue that the ^{14}C depletion is probably a dilution effect arising from the bacterial uptake of low- ^{14}C carbon contained in the DOC pool.

Like the carbon in organic particles, the carbon in the DOC pool acquires a bomb ^{14}C signature when DOC is produced by organisms at the surface. However, the bomb ^{14}C signal reaches the DOC in the interior of the ocean only as fast as the ocean can advect contaminated water into the interior by its relatively slow overturning motions (with a timescale of decades to centuries). Also, the DOC pool is 50–100 times larger than the POC pool and turns over more slowly. Thus, bacteria feeding on DOC incorporate relatively uncontaminated carbon into their cells. As bacteria are eaten and their carbon is incorporated into the tissues of microbial animals, or as bacteria aggregate or otherwise attach themselves onto filterable particles, the low- ^{14}C signature of the DOC dilutes the bomb ^{14}C in the particulate pools. The observations of Druffel and Williams thus appear to confirm that a certain fraction of the particulate carbon in the deep sea has reached the deeper levels of the ocean by the dissolved organic pathway.

Measurements of DOC have been made for over 50 years. But according to Suzuki and Sugimura, the older techniques for oxidizing carbon from the DOC pool extract less than half the total pool⁶. The carbon extracted by the older techniques is also very 'old' with respect to ^{14}C

(around 6,000 years¹⁰), and is thus extremely resistant to microbial oxidation. A product of the old-DOC pool size and its ^{14}C turnover rate suggests that the old DOC is produced at a rate which is only 0.25 per cent of the primary production in the ocean^{4,10}. Much of the old DOC may come into the ocean via rivers¹¹.

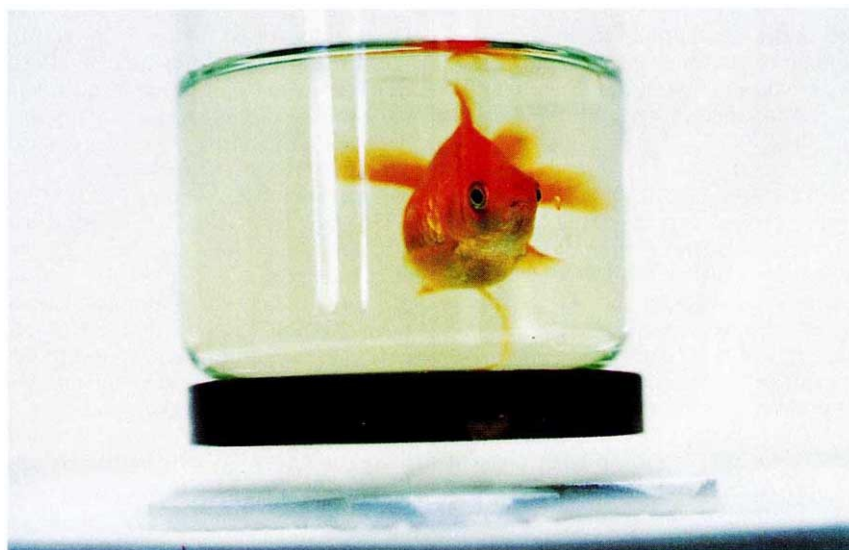
The extra DOC extracted by Suzuki and Sugimura is concentrated in the thermocline and upper ocean⁶. To maintain an upper-ocean DOC gradient against the tendency of the circulation to reduce it, the biota of the ocean must produce the new DOC in much greater quantities than the old DOC is produced. If one assumes that there is an upper-ocean new-DOC pool of roughly 60 moles m^{-2} (a standing crop equivalent to 120 μM spread over the upper 500 m; ref. 6) and one assumes that the upper-ocean pool is turned over in 20–50 years, then the new DOC must be produced at a rate equivalent to 11–28 per cent of the primary production⁷. Production rates of this magnitude, if valid, attest

to a carbon cycle which is dominated by the producers of DOC, whatever they may be¹², and heterotrophic bacteria. As much as three times more carbon may be transported out of the euphotic zone in the dissolved organic form than is transported by sinking particles.

The new DOC must have a ^{14}C age which is similar to that of the inorganic CO_2 in sea water and must be contaminated with bomb ^{14}C , although probably not to the same extent as organic particles. No one has yet succeeded in directly measuring the ^{14}C content of the Suzuki and Sugimura's new DOC. A very powerful statement in support of the dissolved organic pathway in the ocean's carbon cycle awaits proof that the additional DOC extracted by Suzuki and Sugimura contains a much higher $^{14}\text{C}/^{12}\text{C}$ ratio than the DOC extracted by the old methods. □

J. R. Toggweiler is in the Geophysical Fluid Dynamics Laboratory, Princeton University, PO Box 308, Princeton, New Jersey 08542, USA.

Superconducting boost for goldfish



LEVITATING magnets are a familiar sight in superconductivity research. The cause, the Meissner effect — expulsion of magnetic flux lines from a superconducting material — has long been one of the most dramatic manifestations of the superconducting state. But usually the magnets are rather modest — roughly coin-sized — which explains the interest generated by the picture above when it was presented at a recent meeting* by M. Murakami and colleagues from ISTE in Tokyo. The 2-kg goldfish bowl is resting on a rare-earth magnet levitated over a liquid-nitrogen-cooled $\text{Y}_{1.8}\text{Ba}_{2.4}\text{Cu}_{3.4}\text{O}_x$ (YBCO) superconductor.

The levitation in this example is generated by rather more subtle means than the simple Meissner effect. YBCO belongs to

the type II class of superconductors, which permit a degree of penetration by magnetic flux. The flux lines tend to get 'pinned' at defects in the material's lattice. The force holding the bowl up is generated by the resistance to rearrangement of the pinned flux lines, which would result as vertical displacement of the magnet changed the flux density. Its strength is proportional to the sample's critical current and grain size. The advance made by Murakami and colleagues is to prevent cracking of the superconductor's grains by incorporating silver particles in the superconducting matrix: these can absorb mechanical strain without affecting the material's superconducting properties. One hope is that the levitation force may be used to create frictionless bearings, in gyroscopes for example.

P.B.

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* Workshop on high-temperature superconductivity, University of Cambridge, 13–15 August 1990.

Dominant susceptibility genes

G. M. Martin, G. D. Schellenberg, E. M. Wijsman & T. D. Bird

ALZHEIMER's disease is widely recognized as the most common form of dementia in older people, at least in most Western societies. Its prevalence (as determined by a set of clinical diagnostic guidelines, including important exclusionary criteria) increases exponentially as a function of age: a recent survey¹ in East Boston determined that nearly half of those over 85 years old showed signs of the disease. Neuropathologists (upon whom we must rely for a definitive diagnosis) have yet to document any qualitative differences between the several characteristic microscopic effects of the disease and the occasional lesions observed in association with normal ageing. The varying ages for the onset of Alzheimer's disease may simply reflect differing propensities for a common manifestation of ageing in the central nervous system (especially in the hippocampus) — analogous, for example, to the everyday observation that some people seem more susceptible to greying and thinning of the hair than others. If so, what are the precise nature–nurture interactions that underlie such differential susceptibilities?

Linkage assignment

Given the great importance of this question, there is much to admire in the huge collaborative research effort of Peter St George-Hyslop, John Hardy and colleagues, reported on page 194 of this issue², who provide evidence in support of a previously reported³ linkage assignment to the long arm of chromosome 21 for early-onset pedigrees exhibiting autosomal dominant patterns of inheritance of probable Alzheimer's (mean age of onset ≤ 65 years). For the case of late-onset families, however, such a linkage is excluded, as in a previous report⁴. A statistical test comparing early with late-onset families supports the hypothesis of genetic heterogeneity. Other work indicates that there may be genetic heterogeneity even among early-onset Alzheimer's kindreds. Linkage analysis of a subset of families for which there is evidence of a genetic founder effect (Volga German familial Alzheimer's kindreds in which age of onset is approximately 57 years) gives consistently negative results using markers for the same region of chromosome 21 (ref. 5).

How certain can we be of the assignment in early-onset families to chromosome 21? The sceptical observer of the natural history of linkage research for neuropsychiatric disorders will note that there appear to be three contrasting ways that two-point lod scores evolve as additional data accumulate. ('Lod', the log of the odds, is the primary parametric statis-

tical method for the determination of the strength of linkage, a score of 3.0 being the conventional threshold for the acceptance of linkage and a score of -2.0 being the conventional threshold for the rejection of linkage between a polymorphic test marker and the disease locus of interest.) On the one hand, we have the example of linkage of a marker on the short arm of chromosome 4 to Huntington's disease. The lod score for the original US pedigree⁶ was 4.53 at a recombination frequency of 0.0. When these studies were extended to encompass 63 families (mostly Caucasians, but including a few black American and Japanese families), the combined maximum lod score⁷ was 87.69 at a recombination frequency of 0.04. Another example is the linkage of a marker on the long arm of chromosome 19 to myotonic dystrophy, where lod scores have been rapidly increasing, with a recent report of 22.8 at a recombination frequency of 0.03 for the case of 65 families⁸. By contrast, there is the example of bipolar affective disorder in the Amish kindred, in which the original positive score of 4.08 disappeared after the pedigree was updated and extended, the last recorded score⁹ for the 11p marker having become -9.31 .

The new report on Alzheimer's disease² indicates something in between those above extremes — an original score of 2.37 for the first set of four families³ now rising to 4.50 for the extended set of 30 early-onset families (linkage to the DNA haplotype D21S1/D21S11). A multipoint linkage analysis, however, in which the candidate Alzheimer's gene is 'passed' across an interval between two markers of known fixed positions, may lend further support to the hypothesis of a susceptibility gene on chromosome 21.

St George-Hyslop *et al.* also report on the use of a non-parametric method for evaluating the potential for linkage. As noted by the authors of this method¹⁰, however, the method can confound linkage and association. The large number of missing data in the Alzheimer's pedigrees coupled with their ethnic heterogeneity increases the possibility of linkage disequilibrium between the Alzheimer's locus and some of the chromosome 21 markers in the sample. Therefore, the results need not indicate a *bona fide* genetic linkage. The method cannot be regarded as a confirmation of linkage, which would require the analysis of an independent, unselected set of early-onset pedigrees. But a perfectly reasonable proposition is that alleles at a locus on chromosome 21 may indeed contribute to susceptibility to Alzheimer's in at least some early-onset families.

What tentative conclusions can now be

made regarding the genetic substrate for the clinically more significant and much more difficult¹¹ problem of late-onset Alzheimer's? The latest report² tells us only that there is no evidence for a comparable susceptibility gene in chromosome 21. It leaves open any number of possibilities, including the interaction of multiple minor genetic effects with multiple environmental factors. Recent research (ref. 12; M.A. Pericak-Vance *et al.*, submitted to *Am. J. hum. Genet.*) using the same set of parametric and non-parametric methods as St George-Hyslop *et al.* provide "highly suggestive" evidence for a linkage assignment to the proximal long arm of chromosome 19.

Linkage analysis

What of the future? Can the now tried-and-true approach of identifying additional more closely linked markers followed by chromosome walking and cloning be used to identify the first familial Alzheimer's gene? The spectacular successes with cystic fibrosis¹³ and neurofibromatosis¹⁴ were based on narrowing the possible loci to a genetic distance equivalent to 1–2 million base pairs using linkage analysis. If early-onset familial Alzheimer's is genetically heterogeneous, linkage analysis may give only a regional localization, perhaps as large as 20–30 million base pairs. If this is so, new approaches for identifying all the genes in a large region and screening those candidate genes for Alzheimer's mutations will have to be developed.

It will also be vital to pursue independent approaches, particularly efforts to document a phenotype in cultivated non-neuronal somatic cells. We should recall that this strategy led to the breakthrough (and a Nobel prize for Joe Goldstein and Michael Brown) on the pathogenesis of atherosclerosis, another common, late-onset disorder, the complex pathogenesis of which may serve as a model for Alzheimer's research¹⁵. There are some hints that this might be feasible¹⁶. □

The authors are associated with the Alzheimer's Disease Research Center, University of Washington, Seattle, Washington 98195, USA.

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Diamonds are for everywhere

Joe Nuth

DIAMONDS and silicon carbide grains older than the Solar System were once present in virtually all chondritic meteorites in roughly equal proportions, according to a paper by Gary Huss on page 159 of this issue¹. The abundance of these pre-solar materials is directly correlated to the fraction of fine-grained 'matrix' in the meteorite and is therefore inversely related to the degree to which the meteorite was processed in its parent body. Primitive (unprocessed) meteorites such as Orgueil, Semarkona and Vigarano contain approximately 1,000 parts per million (p.p.m.) diamond and a few p.p.m. SiC per gram of matrix material, even though the fraction of matrix in these individual meteorites varies by roughly a factor of six.

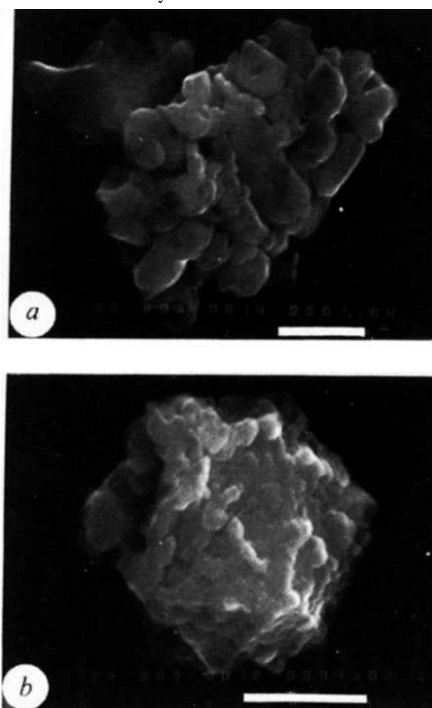
Even more remarkable is the similarity in the isotope composition of the noble gases (especially Xe) released during pyrolysis from the diamonds recovered from widely differing meteorite types. These observations indicate that the matrix of primitive chondritic meteorites may contain a relatively unbiased sample of the refractory interstellar grain population which existed at the time our Solar System formed nearly five billion (10^9) years ago.

Until only very recently most meteoritists firmly believed that virtually all interstellar grains once present in the giant molecular cloud which collapsed to form our Sun were destroyed before the planetesimals, parents to the planets, formed. Grains were expected to vaporize when heated to the very high temperatures thought to exist in the pre-solar nebula. Even if a few grains had managed to survive the hot nebula, the processes by which planetesimals became compacted and heated, then fractured by collisions in the asteroid belt, long before their host meteorites found their way into our terrestrial laboratories, were sure to eliminate all traces of a grain's interstellar heritage. Several remarkable discoveries over the past several years have shown that there are flaws in this version of pre-solar history.

Although it was recognized as early as 1969 that some samples of noble gas extracted from meteorites could have been trapped in grains formed in novae², it was only after the isolation of the carriers of these anomalies — diamond³, SiC⁴ and graphite⁵ — by Anders and his co-workers that it became clear that relatively pristine interstellar grains could be preserved in some meteorites. But until the new work by Huss¹ there had been no systematic survey of meteorite types for evidence of pre-solar materials: the grains recovered by Anders and co-workers might have

been isolated and very rare instances in which pre-solar materials had been inexplicably sequestered and preserved. These grains would say little about either interstellar grains or conditions in the pre-solar nebula if they were simply odd quirks of fate.

Huss¹ clearly demonstrates that it was



Interstellar silicon carbide grains from the Qingzhen meteorite. *a*, Aggregated with spinel (the di-pyramidal crystal, top centre; the large spinel grain, top left is probably not part of the aggregate). *b*, Isolated grain. Scale bars, 1 μ m. (Courtesy of G. Huss.)

not at all unusual for interstellar grains to survive the conditions in the solar nebula. He also demonstrates that both SiC and diamond grains were readily destroyed by relatively mild metamorphic processes in the meteorites' parent bodies. Furthermore, the similarity in the isotopic composition and release pattern of anomalous noble gases from a large number of chondritic meteorites indicates that, although individual SiC or diamond grains may have been formed in specific circumstellar environments, grains from a variety of sources seem to have been thoroughly homogenized before they were incorporated into meteorites. These grains are best preserved in the most primitive meteorites.

What might this mean for meteoritics? First, the relative depletion of circumstellar diamond, SiC, or other forms of easily identified carbon such as graphite, hydrogenated amorphous carbon or diamond-like hydrocarbon, might be used

as a very sensitive cosmo-thermometer with which to measure the maximum temperatures reached by individual meteorites. Such a cosmo-thermometer should be quite easy to calibrate in the laboratory by heating samples of a primitive meteorite such as Orgueil, under vacuum for various times at a number of temperatures and then measuring the depletion of specific noble gas components in acid residues.

A corollary to this research would be a measure of the thermal and chemical stabilities of the specific noble-gas carriers themselves in hydrogen and under vacuum, to set limits on the maximum temperature which might have been achieved by the nebular gas and dust before the chondrites formed. This maximum would not apply to transient events which may have produced refractory phases, such as the spherical chondrules and calcium-aluminium inclusions (CAIs) found in chondrites, in isolated regions of the nebula. But it would place an upper limit on the base temperature of the system.

It may be that the relative proportions of chondrules, CAIs and matrix are more a measure of the power and frequency of any transient events that accompanied the formation of the meteoritic parent body, than of the temperature of the nebula as a whole. If this is true then it would help to reconcile the disparity between the low temperatures observed in modern protostellar nebulae and the very high temperatures required to form the refractory inclusions observed in meteorites.

Researchers might also use the availability of a nebular or meteorite cosmo-thermometer to assist the search for a sample of interstellar silicate grains by carefully examining the silicates from the matrix material of the chondrite which has been heated least and which contains the largest fraction of matrix. Potential indicators of interstellar materials might include carbonaceous coatings on grains of unusual isotopic composition or the direct physical association of a grain with carbonaceous materials of known interstellar origin such as diamonds or SiC. If one were to compare the chemistry and morphology of grains found under such circumstances with grains in the matrix of unheated chondrites which contain large fractions of chondrules and CAIs, then it may be possible to distinguish pre-existing interstellar silicates from those condensed in the solar nebula.

Because the average composition of chondritic meteorites is approximately constant despite the proportion of refractory inclusions, vapour distilled during the formation of these inclusions must have recondensed quite rapidly and been incorporated into the matrix fraction of the same parent body. Therefore a relatively high proportion of refractory inclusions in

the meteorite should indicate a larger fraction of nebular condensate in the matrix. Identification of nebular condensates would provide very sensitive indicators of the redox state of the nebula as such grains are extremely small and would equilibrate rapidly with the nebular gas.

Finally, if all primitive meteorites once contained a fairly complete sample of the interstellar grain population there can be no doubt that comets also contain such a record. What better place to search for a truly unbiased sample of interstellar materials than in the frozen interior of a comet? Such samples might preserve not only the refractory grains found in meteorites, but the more volatile components as well. From meteoritic samples cosmochemists have already obtained direct confirmation of previously hypothesized nucleosynthetic processes.

HAEMATOPOIESIS

Searching for stem cells

Norman Iscove

FOUR decades have passed since the discovery that the injection of intact marrow cells could rescue lethally irradiated rodents and permanently replace their haematopoietic and lymphoid organs with donor cells. It is therefore rather surprising that the nature and properties of the 'stem' cells in marrow responsible for long-term reconstitution remain the subject of disagreement. The study by Jones *et al.* reported on page 188 of this issue¹ represents a new turn in the long road to the identification of stem cells.

The chief difficulty in defining the stem cell has been its rarity in haematopoietic tissues. Early efforts at morphological definition were not notably productive, and real advances had to await the advent of the spleen colony assay in 1961². The method entailed the intravenous injection of marrow cells into heavily irradiated mice. Cells called CFU-S, for spleen colony-forming units, were found to be capable of lodging in the spleen and forming macroscopic nodules within 7 to 12 days of injection. The nodules were shown to derive from single cells, and frequently consisted of differentiating cells in several haematopoietic lineages. Moreover, colonies contained varying numbers of CFU-S which were again able to generate spleen colonies on injection into secondary irradiated hosts. Based on this evidence, CFU-S seemed to possess the key capabilities of proliferation, pluripotentiality and self-renewal considered to be the hallmarks of stem cells. It was irresistible for many to conclude that CFU-S and cells responsible for long-term reconstitution were the same.

Despite the appeal of CFU-S as the ultimate stem cells, further observations

From materials obtained by the European Space Agency and NASA's Comet Nucleus Sample Return mission, proposed for the turn of the century, future generations may be able to unravel the details of the nucleosynthetic process to a degree not previously thought possible, not to mention the myriad other studies made possible by access to a large sample of relatively pristine interstellar material. □

Joe Nuth is at the NASA Goddard Space Flight Center, Greenbelt, Maryland 20770, USA.

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were difficult to reconcile with that view. Only a few spleen colonies contained significant numbers of CFU-S, and extended serial passage was impossible. Although the findings were consistent with stochastic self-renewal by initially equivalent stem cells, it was found as early as 1969³ that those CFU-S with the greatest self-renewal capacity could be physically separated from the others, giving the first indication that CFU-S might be heterogeneous. Much later, it was shown directly that day-12 CFU-S (enumerated by counting colonies at day 12) were distinct from, more primitive than and more highly self-renewing than those assayed earlier⁴.

Other discrepancies emerged from experiments that more directly examined the properties of reconstituting cells. Early work established their capacity to reconstitute the lymphoid as well as the haematopoietic systems^{5,6}, and to feed both for periods now known to extend for at least 15 months⁷ in mice. If spleen colonies were derived from such cells, then the colonies should contain lymphoid cells or their precursors, as well as cells capable of long-term multilineage reconstitution *in vivo*. But direct tests of spleen colonies were negative for both kinds of cell. Reconstituting stem cells also differed from CFU-S in their insensitivity to 5-fluorouracil⁸, a drug that kills cycling cells, and in their limited capacity to regenerate themselves after transplantation into irradiated mice⁹. These observations demanded recognition of significant heterogeneity in the population detected in the CFU-S assay, and seemed to relegate ability for long-term reconstitution to a distinct minority of them.

The art of cell purification has advanced significantly in recent years, and it should now be possible definitively to define the relationship between CFU-S and the reconstituting stem cells. Spangrude *et al.*¹⁰ purified early precursors on the basis of surface antigen expression (low level of Thy-1 and absence of lineage-specific markers — that is, phenotype Thy-1^{lo} Lin[−]) and subdivided them on the basis of Sca-1 (Ly6A.2) expression. About half of the day-12 CFU-S were segregated into the Thy-1^{lo} Lin[−] Sca-1⁺ fraction, and comprised most of the fraction as inferred from the high yield of spleen colonies. When tested for reconstituting activity in irradiated mice, only 30 purified cells were needed to rescue 50 per cent of mice from lethal irradiation, and multilineage lymphoid and myeloid reconstitution was achieved in a mouse that received only 40 cells. The implication was that a substantial fraction of marrow day-12 CFU-S, perhaps as high as half, might represent multilineage reconstituting stem cells. However, the work remained inconclusive: radiation survival is not necessarily synonymous with long-term reconstitution by donor cells in such assays because, given help, the numbers of host stem cells surviving the irradiation are usually sufficient to regenerate the haematopoietic tissues. Moreover, it was still possible that the reconstitution from donor cells documented in a single mouse arose only from a minority subpopulation in the Sca-1⁺ day-12 CFU-S fraction. And finally, without quantitative measurement of reconstituting cells in all fractions, it was not possible to assess the proportion that were present in the Thy-1^{lo} Lin[−] Sca-1⁺ fraction as opposed to other fractions.

If the study from Spangrude *et al.*¹⁰ suggested substantial overlap of day-12 CFU-S and multilineage reconstituting cells, the more recent report from Ploemacher and Brons¹¹ seemed to place more restrictive limits on the overlap. These authors enriched marrow precursors on the basis of sedimentation velocity and light-scattering properties, and then fractionated them according to their retention of radioactive rhodamine-123. The fractions were assayed both for day-12 CFU-S as well as for their precursors, defined by their ability to regenerate numbers of day-12 CFU-S in the marrow 13 days after injection. Strikingly, the fraction with 90 per cent of the pre-CFU-S activity separated from more than 97 per cent of day-12 CFU-S.

The study by Jones *et al.*¹ now makes this demarcation even clearer. Separating marrow cells on the basis of sedimentation velocity, these authors took the care to assay all fractions not only for CFU-S, but also for ability to protect irradiated recipients from radiation death and to repopulate the marrow with donor cells at early (14-day) and late (60-day) time points.

Intermediate and rapidly sedimenting fractions contained 99.8 per cent of the day-12 CFU-S as well as all the cells capable of radiation protection and short-term reconstitution. In striking contrast, marrow reconstitution that lasted 60 days came predominantly from a slowly sedimenting cell fraction that contained only 0.25 per cent of the day-12 CFU-S and none of the protective activity. The study of Jones *et al.* is technologically modest, attempting neither extensive phenotyping of the stem cells nor even significant enrichment, yet the results have much to teach us. First, the extent of possible overlap of day-12 CFU-S and long-term-reconstituting units appears to be very low indeed and seems incompatible with the conclusions drawn by Spangrude *et al.*¹⁰. Second, sedimentation velocity may be the strongest discriminator so far described between these early precursor populations. Third, cells responsible for short-term radiation protection and those capable of longer-term reconstitution are physically distinct. Finally, the assessment of the relationship between cells detected in different assays requires determination of the distribution of the relevant biological activities across all of the fractions in a separation experiment.

The study of Jones *et al.* seems to bring a formative era in experimental haematology to a close. It is now clear that a very sizeable majority of CFU-S, for practical purposes perhaps all of them, are distinct from the stem cells responsible for long-term maintenance of haematopoiesis. CFU-S seem to be a heterogeneous population of more advanced precursors, arguably studied with greater precision by *in vitro* methods¹², and now of interest mainly for their importance in founding the modern approach to analysis of precursors by clonal methods. For the cells of greatest practical interest — those with long-term reconstituting ability — there still seems to be no satisfactory substitute for long-term assays *in vivo*^{7,9,12}. □

Norman Iscove is at the Ontario Cancer Institute, 500 Sherbourne Street, Toronto, M4X 1K9, Canada.

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Multiphoton laser cooling

Andrew Steane and Christopher Foot

THE force exerted on an atom by low-intensity laser beams has proved to have many interesting and useful properties, and the laser cooling and trapping of atoms using this radiation force is becoming common. Less work has been done

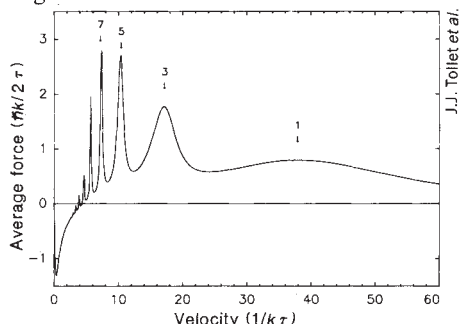


FIG. 1 Predicted force–velocity curve for the multiphoton or Doppleron interaction of an atom with a standing wave. The great width of the single-photon peak is attributable to ‘power broadening’, a result of the high laser intensities used in such experiments.

with high-intensity beams, but two recent papers (N. P. Biglow & M. G. Prentiss *Phys. Rev. Lett.* **65**, 555–558 and J. J. Tollet *et al. Phys. Rev. Lett.* **65**, 559–562; 1990) have begun to confirm the theory of this case and to explore the experimental possibilities. The interest of the work lies in the clear identification of multiphoton processes and in the possibility of exerting forces on an atom much larger than is possible by single-photon scattering.

An atom in a light field will absorb photons, and emit them both spontaneously and under the stimulation of the radiation field. Each time a photon is absorbed or emitted, its momentum $\hbar k$ is imparted to the atom in the relevant direction, where k is the wave vector of the light ($k=2\pi/\lambda$ for wavelength λ) and $\hbar$ is Planck’s constant. For example, for an atom in a single travelling wave each absorbed photon gives the atom a kick along the direction of the wave and photons emitted by stimulated emission impart a kick in the opposite direction; the spontaneously emitted photons go in random directions and produce no net force. The overall force is, therefore, $\hbar k$ multiplied by the rate at which photons are absorbed (minus the stimulated emission rate) which is simply equivalent to $\hbar k$ times the spontaneous emission rate. As the intensity increases this force saturates at a maximum value of $\hbar k/2\tau$, as the spontaneous emission rate is limited by the lifetime τ of the excited state. The atom spends half the time in the excited state when the transition is saturated.

To obtain forces larger than this maximum, more than one travelling wave must

be used. One then has the possibility of absorption from one wave followed by stimulated emission into another wave, with a net change in momentum of the atom. This stimulated process will not saturate and so can produce a force larger than the maximum scattering force. Both of the new papers consider the case of atomic motion along a pair of counter-propagating travelling waves which form a single standing wave. The theory of the atom–light interaction in this arrangement was originally developed to explain phenomena related to saturation in gas lasers. With the advent of laser cooling, calculations were made of the force exerted on an atom as a function of the velocity of the atom along the standing wave (Fig. 1).

A distinctive feature of the force–velocity curve in these circumstances is the appearance of several sharp peaks in the force at various velocities. This can be understood as follows. If the frequency ω_L of the laser producing the standing wave is somewhat lower than the atomic resonance frequency ω_0 , then a stationary atom is not in resonance with either of the two travelling waves. An atom moving with a velocity v , however, will experience the two waves Doppler-shifted by $\pm kv$. When the Doppler shift just compensates for the detuning of the laser, that is $kv = \omega_0 - \omega_L$, the atom is just in resonance with one wave but not with the other and scatters more photons from one direction than from the other. This single-photon process is simply the scattering force and produces a power-broadened peak limited as before to a maximum value $\hbar k/2\tau$.

At lower velocities the atom interacts

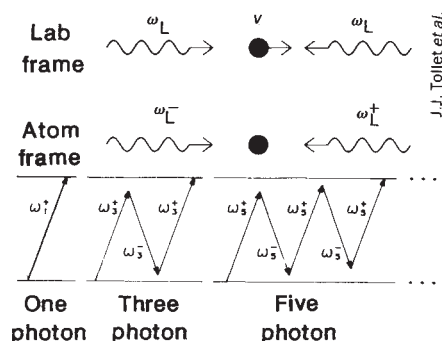


FIG. 2 The Doppleron mechanism. Upper, atoms moving at velocity v through a standing wave radiation field (ω_L) see the radiation at two frequencies, one greater (ω_L^+) and one less (ω_L^-) than the laser frequency (middle). With the laser tuned appropriately, the atoms can be made to absorb the detuned photons in a series of steps, each a forced resonance through a Raman-type (or ‘virtual’) state (bottom).

with the waves by multiphoton processes with several stages of absorption and stimulated emission, in which the momentum kicks imparted to the atom are all in the same direction (Fig. 2). For example, a three-photon stimulated Raman process involving the absorption of two photons from one wave and stimulated emission of one photon into the other will be resonant when $k\nu = (\omega_0 - \omega_L)/3$. There is a coherent redistribution of photons between the waves, involving a momentum exchange of $3\hbar k$, so that the force due to this process can rise to three times the single-photon maximum. Further odd-order processes involving n steps are resonant at velocities $k\nu = (\omega_0 - \omega_L)/n$ and produce narrower peaks, because the 'power broadening' becomes weaker. Eventually the highest-order processes become insignificant owing to their low probability. The term Doppleron has been coined for these resonances, because they can be regarded as an absorption process of n Doppleron quanta of energy $\hbar k\nu$ as seen by the atom.

LUNAR ORIGINS

Experiment confronts theory

Michael J. Drake

AFTER countless years of speculation (much of it idle), data from lunar and planetary missions have allowed space scientists to get a clearer picture of the way the Moon was formed. But as the new geochemical data presented by Ringwood and co-workers on page 174 of this issue¹ show, the idea that the debate is over is far from the truth.

Before the Apollo missions to the Moon, three theories of lunar origin held sway: the capture theory, according to which the Moon formed independently from the Earth and was gravitationally captured by it; the co-accretion theory, which held that the Moon formed simultaneously with the Earth from material in orbit about the Earth; and the fission theory, which held that the Moon was derived from the mantle of the Earth after core formation through rotational instability. As a result of the Apollo missions, we know that the Moon is deficient relative to the Earth in volatile elements. Even before the Apollo missions, we knew from the low mean density of the Moon (3.344 g cm^{-3} , almost identical to that of the Earth's mantle) that the Moon is depleted in metallic iron compared to the Earth. But these facts did not provide convincing evidence in favour of or allow for the exclusion of any one theory. Rather, we learned a great deal about the evolution of the Moon and, hence, the early history of the inner Solar System. The Moon has become a touchstone for understanding the cratering record of the

The new experiments have revealed such multiphoton processes up to seventh order and the forces achieved can be a few times the maximum scattering force. An accurate test of the theory, which includes some approximations not really valid for the experiments, is still to come. Also, so far the total atomic deceleration has not been sufficient for the method to produce a useful source of slow atoms. For this purpose an interesting prospect is to emulate the 'chirping' technique used in laser cooling with the scattering force. One would use a pair of travelling waves with slightly differing frequencies to make a 'standing' wave that actually moves. By sweeping the frequency difference, one could allow the Dopplerons to keep in step with the slowing atoms and so cool a thermal atomic beam efficiently. Although this has been attempted by a few groups, no success has yet been reported. □

Andrew Steane and Christopher Foot are in the Department of Physics, University of Oxford, Parks Road, Oxford OX1 3PU, UK.

inner Solar System because it is the only body in the Solar System which has both preserved its bombardment record and had rocks dated from known surfaces.

Following Apollo, two additional theories of lunar origin were proposed: the disintegrative capture hypothesis which held that the Moon formed in Earth orbit from matter which formed elsewhere (but in the vicinity of our orbit) and had been disrupted as it passed through the Earth's gravitational 'Roche' limit (well within 2.5 Earth radii) and had lost its metal; and the giant-impact or collisional-ejection hypothesis which held that the Moon was formed by collision of a Mars-sized object with the Earth late in the accretionary stages of the Earth.

The giant-impact hypothesis has since become the paradigm for lunar origin^{2,3}, in part because computer simulations of the accretion of the terrestrial planets predict that many bodies as large as the Moon and Mars would exist in the vicinity of our orbit towards the end of the period of accretionary planetary growth. The giant impact of such a body with Earth could account for the net angular momentum of the Earth-Moon system, provided that the blow was a grazing or glancing one. But there is still the question of whether the Moon was made from the remnants of the impacting object or whether debris from the Earth's mantle blasted into orbit by the impact merged to form the Moon. According to the computer calculations⁴, at least half if not all of the Moon came

from the impactor's mantle.

Ringwood, on the other hand, has long argued that the Moon is derived largely from the Earth's mantle⁵. The basis for his arguments include the low density and lack of volatile elements in the Moon, but arise also from certain compositional similarities of the Moon and the Earth's mantle. In this issue¹, Ringwood and co-workers point to the similarities in the abundances of vanadium, chromium and manganese in the mantles of the Earth and Moon (an observation for which there is a broad consensus) and ask how such similarity might have come about.

Specifically, abundances decrease in the order $V > Cr > Mn$. Earlier, my colleagues and I had shown⁶ that segregation of metal from silicate during the formation of the Earth's iron-nickel core would impose an abundance pattern of V, Cr and Mn in the Earth's mantle different from the one observed, if partitioning of these elements between metal and silicate is as determined experimentally at 1 bar (one atmosphere) pressure. Ringwood and co-workers have conducted experiments at 160 kbar. These experiments still do not show that the V, Cr, and Mn abundances in Earth's mantle were established by equilibrium between metal and silicate as the core segregated from the mantle: the partitioning of oxygen-bearing metallic liquid from silicate liquid at 160 kbar would impose the same pattern on the mantle as deduced from experiments at 1 bar. Experiments at megabar pressures (more typical of the terrestrial core-mantle boundary processes) could still indicate a different partitioning pattern.

But Ringwood and co-workers do show that core-mantle equilibrium in a Mars-sized object will not yield the abundances of V, Cr and Mn observed in the mantles of the Earth and Moon. Indeed, if the 'SNC' meteorites are from Mars, the mantle of Mars itself has very different abundances of V, Cr and Mn from the terrestrial and lunar mantles.

Nevertheless, Ringwood and co-workers' conclusion that the Moon is derived substantially from the Earth's mantle, presumably by equilibration of core and mantle at substantially higher pressures than experimentally investigated thus far, also faces problems. For example, although similar, the abundance of volatile Mn is higher in the Moon than in the Earth, opposite to what would be

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expected to result from energetic events that depleted the Moon in all other volatile elements relative to the Earth. Furthermore, the Rb/Cs ratio in the Earth's mantle is higher than that of the Moon⁷. Caesium, however, is more volatile than Rb, and the terrestrial Rb/Cs ratio should be the same as or lower than the Moon's. Finally, the ⁴⁰K/³⁹K ratio in lunar feldspars is lower than in the terrestrial feldspars⁸. If the Moon were derived substantially from the Earth's mantle, the ratio would be expected to be the same or higher, as the lighter isotope would be preferentially lost in any energetic process responsible for the depletion of volatile elements in the Moon relative to the Earth.

CONSERVATION BIOLOGY

Taxonomy as destiny

Robert M. May

THE tuatara is a large, iguana-like reptile whose main claim to fame is as the sole survivor of a once-flourishing group whose heyday was the Triassic Period, more than 200 million years ago. Daugherty *et al.*, on page 177 of this issue¹, suggest that the greatest threat faced by the tuatara, which is now confined to a few islets off the coast of New Zealand, is a persistent failure to recognize that populations of the animal are genetically far more diverse than legislators have cared to admit.

Completely unrelated to the superficially similar lizards, the tuatara has a well-developed third eye in the centre of its head (most other vertebrates retain this organ in vestigial form as the pineal gland, behind the third ventricle of the brain) and a row of yellow spines down its back (hence the name, from the Maori *tua*, 'on the back'; and *tara*, 'spine').

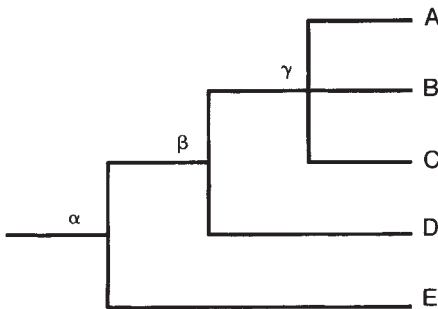
As Daugherty *et al.*¹ point out, three species of tuatara were recognized and named in the 19th century. One is now extinct. A second, *Sphenodon guntheri*, has suffered the curious fate of being ignored by the legislation designed to protect the tuatara, which has only ever acknowledged a single species, *S. punctatus*. This discrepancy dates back to the 1895 Animals Protection Act in New Zealand, even though *S. guntheri* had been described and recorded as a distinct species in 1877. Daugherty *et al.* argue that this legislatively-defined monotypy of the tuatara has affected its destiny, engendering a belief that 'the species' is relatively widespread and the documented extinction of 10 out of a total of 40 populations over the past century is not too serious.

Daugherty *et al.* report allozyme variation and morphological differences from 24 of the 30 islands on which tuatara populations are thought to survive — studies

Until positive proof is obtained that the abundances of V, Cr and Mn in the Earth and Moon would be the result of megabar metal/silicate partitioning processes acting deep inside the Earth, the idea that the Moon was derived substantially from the Earth remains a theory. Ringwood and co-workers have done the scientific community a service in reminding us that bandwagons are dangerous things⁹ and that the giant-impact theory for the origin of the Moon does not readily explain all chemical and isotope parameters with sufficient robustness for the matter to be closed. □

Michael J. Drake is in the Lunar and Planetary Laboratory, University of Arizona, Tucson, Arizona 85721, USA.

that unequivocally support the status of *S. guntheri* as a distinct species. At present fewer than 300 individuals of this species exist, confined to just one island, North Brother Island in Cook Strait; one other



A hierarchical classification or tree for 5 imaginary species, labelled A, B, C, D and E. The symbols α , β and γ label the nodes or branching points in the tree.

population became extinct earlier this century. There are clear implications for conservation management on North Brother Island. In practice, intervention and management on behalf of any endangered population of tuatara has been limited, and contrasts with successful conservation techniques applied to some threatened species of birds in New Zealand. On four islands where existing populations of *S. punctatus* are currently on the brink of extinction, endangered birds have been deliberately introduced and sometimes intensively managed, possibly to the detriment of the tuatara.

The new work¹ raises larger questions about the ways in which relative values are assigned to different creatures — about how we weigh, as it were, taxonomic distinctness. As more and more species face extinction in the wild over the next few decades, how do we go about making choices for the ineluctably limited number of places on the ark? For a given group of

organisms, or more generally, how do we go about designing a limited set of reserves that will, in some defined sense, optimize what is saved?

To be specific, how might we value the tuatara, be it one species or two, against any other species of reptile? At one democratic extreme, it could be argued that all species are to be valued equally, each a unique evolutionary product. But does a conservation biologist regard the tuatara — questions of relative abundance set aside for the moment — as equal to any other one or two species of skinks or grass-snakes? Could the World Wide Fund for Nature replace its panda logo with any one species of rodent?

At the opposite extreme, Vane-Wright *et al.*² propose that we might consider turning to the taxonomy of the group, expressed as a hierarchical 'tree', and give equal weight to each 'sister group'. Sister groups may be roughly defined as those ultimately having branched from a common node at some point in the hierarchical tree: for example, in the figure, species E and the set of four species A, B, C and D are sister groups (sharing the node labelled α); similarly, the groups D and A, B, C are sisters (from β); and so on. On this basis, the two living species of tuatara would be weighed equally with the sum of all 6,000 species of snakes, lizards and amphisbaenians (which are like the As, Bs and Cs to the tuatara's E in the complex real version of the tree for all reptiles).

Clearly, a sensible intermediate between these two extremes is required, to give some precision to Atkinson's commonsense observation³ that "Given two threatened taxa, one a species not closely related to other living species and the other [a] widespread and common species, it seems reasonable to give priority to the taxonomically distinct form". At recent meetings in Bergen and Canberra on biodiversity, R. I. Vane-Wright, C. J. Humphries and P. H. Williams (from the Natural History Museum, London) discussed these problems, proposed several possible solutions, and explored the implications of measures of 'taxonomic distinctness'. The work has been submitted for publication⁴ under the title "What to protect? Systematics and the agony of choice".

Vane-Wright *et al.* base their ideas on the information content of the topology of a particular hierarchical classification. The broad outlines of their scheme, however, can be grasped without reference to the underlying details. For each species, or twig tip, we trace down to the root of the tree, counting nodes. Thus, in the figure, we count three nodes (α , β , γ) for species A, B and C, two for species D, and one for species E. The species are then each given an index of taxonomic distinctness that is inversely proportional to this node-count. Scaling the minimum such

index to 1, species A, B, C, D and E then have the relative weights 1, 1, 1, 1.5 and 3, respectively. This is clearly different from weighting all five species equally, although in this excessively simple example it is not too different from the extreme of weighting sister groups equally (giving relative weights 1, 1, 1, 3 and 6 to A, B, C, D and E respectively).

This particular scheme of Vane-Wright *et al.* has the slight disadvantage that, for the values accorded individual species, it rests solely on topology, taking no account of how many lines branch from each node. A minor refinement, retaining the spirit of their ideas, is to count not simply the number of nodes between twig-tip and root, but rather the sum of the branches at all such nodes. In this scheme, the nodes labelled α , β , γ in the figure have 2, 2 and 3 branches respectively, and consequently we count 7 for A, B and C; 4 for D, and 2 for E. Taking these numbers to give inverse measures of taxonomic distinctness, as before, we arrive at the relative values of 1, 1, 1, 1.75 and 3.5 for A, B, C, D and E respectively. The differences from Vane-Wright *et al.*'s scheme are small here, and disappear altogether in fully resolved trees, but can be significant in partially resolved hierarchies in which some branches are binary whereas others are multifoliate.

Returning to the tuatara, if all species are weighted equally, the two species represent only 0.03 per cent of all reptiles. At the opposite extreme — equal weight for all sister groups — they represent 50 per cent. Depending on how one approximates the complex topology of the hierarchical classification of all reptiles, I estimate that the scheme developed by Vane-Wright *et al.* implies that tuataras represent between 0.3 and 7 per cent of the taxonomic distinctness found among the 6,000 reptile species (and probably closer to the lower number).

Lifting our sights from species to faunal assemblies, suppose we wish to find, from some larger list, the minimal set of reserves that will include, say, every one of the 158 species of milkweed butterflies. In its simplest form, this is a version of the travelling-salesman problem². Ackery and Vane-Wright⁴ have shown that, in practice, a good solution is obtained by examining a compendium of some 350 sample areas distributed around the globe, and noting the numbers of species (and patterns of endemism) in each area. They then work up a solution by first choosing the most species-rich region, which is the island of Sulawesi with 33 of the 158 (9 of which are endemic). Next comes the region with the highest remaining total, excluding the species already present in Sulawesi. And so on, always choosing the best region to compensate for species not yet incorporated.

In this way, Ackery and Vane-Wright

find that a total of 31 reserves provides a critical set that embraces all species of milkweed butterflies. Although, as Vane-Wright *et al.* point out², this simple method does not necessarily give the minimal set, it is a sensible and efficient method that is surely close to, if not exactly, optimal. A much more complex example of this kind is the recent assignment of priority-grades to different areas of Amazonia, according to measures of the richness of plant and animal species⁵.

The above-mentioned studies were essentially based on counting species. Were it not possible to acquire a range of reserves that included all the species in the designated group, the criterion would presumably be to maximize the attainable number. But if we weigh the species according to some measure of taxonomic distinctness, along the lines just developed, the answers can be significantly different. Vane-Wright *et al.* illustrate this by first using the topology of a current classification of the bumble-bees in the *Bombus sibiricus* group to assign indices of taxonomic distinctness to each of the 43 species in question, and then carrying out an analysis of priority areas among the 120 equal-area grid squares occupied by members of this group on a global map. By a simple species count, the top-ranking grid square centres on Ecuador, with 10 species (23 per cent of the total). But when taxonomic distinctness is taken into account, the top square lies in the Gansu area of China, containing 23 per cent of the total weighted scores for taxonomic distinctness, versus 15 per cent for the Ecuador-centred square.

All this work represents only the beginnings of what might be called the calculus of biodiversity. At very least, we need to combine quantitative measures of taxonomic distinctness with more familiar ecological considerations of abundance and geographical distribution^{6,7}. Some may find it surprising that taxonomy, embodied in the hierarchical trees of systematists, stands alongside ecology in the emerging calculus. There should be no surprise. Without taxonomy to give shape to the bricks, and systematics to tell us how to put them together, the house of biological science is a meaningless jumble. □

Robert M. May is in the Department of Zoology, University of Oxford, South Parks Road, Oxford OX1 3PS, UK.

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Tame twisters

SOLAR energy, says Daedalus, is usually too dilute and diffuse to be harnessed easily. A tornado, however, gathers it from a very wide area. The Sun-heated land of the region warms the air just above it; this low-lying stratum of warm air, seeking to rise, forms a large convection cell with a small central rising column. All the heat energy of the indrawn air, and its angular momentum from the Earth's rotation, is concentrated in this narrow updraught, which spins and rises violently. Daedalus is now devising a 'tame tornado', capable of generating useful power.

A wild tornado, of course, wanders destructively about. Daedalus's tame one will be safely anchored to one spot. Its central rising column will be defined and located by a tall chimney. At first the engineering problems looked daunting. Some sort of vast cooling tower seemed called for, many hundreds of metres tall, supported clear of the ground to admit radially intruding air, and strong enough to withstand its violent spin and upflow inside. But Daedalus then recalled the airport windsock, that simple fabric sleeve kept extended and inflated by the wind blowing through it. So his tame tornado is now simply an outside vertical windsock. Like its airport counterpart, its inlet will be kept circular by attachment to a rigid hoop, anchored by cables to a single axial point. The air rising within it will keep it inflated and vertically extended; the cable anchorage will stop it blowing away upwards, but will allow hoop and sock to spin freely about their vertical axis. Internal 'sails' on the windsock will act as turbine blades driven by the spinning air column within. Thus the whole windsock will spin with the twisting updraught inside it, delivering useful power to a generator attached to the anchorage.

The tame tornado will be activated whenever the weather seems promising. The process will be something like launching a big fire balloon. The windsock will be inflated by a powerful draught of warm air, blown by big ducted fans and heated by propane burners, until natural tornado convection takes over and the windsock is self-sustaining. Thereafter, the installation will run for days or weeks on end, capturing much of the solar energy over a wide radius of countryside. Even at night, the Sun-heated soil will keep the convection cell in being.

A tame tornado will handle much less energy than the wild variety, and should be a relatively mild and safe device. Nonetheless, a grid of them spread across tornado country would usefully steal the energy needed by wild tornadoes. Any tornado approaching the grid would find itself rapidly starved of power, and would soon collapse exhausted.

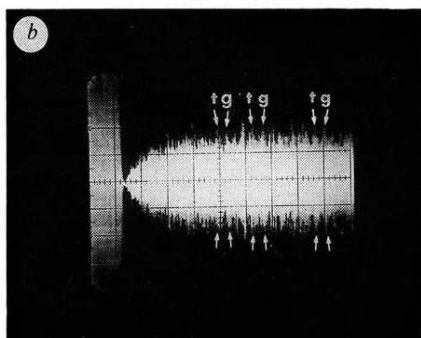
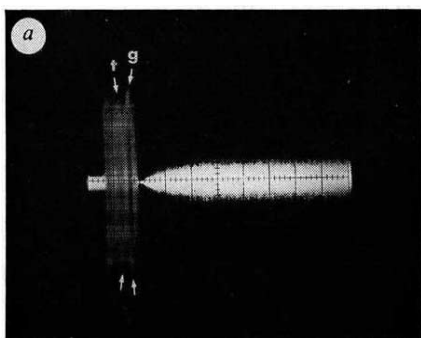
David Jones

An unusual long-delay echo

SIR—Long-delay echoes (LDEs) are weak signals heard at times ranging from several hundred milliseconds to several seconds after an initial radio transmission. They were first reported in the late 1920s (refs 1–3) and have often been reported since (see ref. 4). But they are elusive, and might be considered as an example of “pathological science”⁵.

I carried out a search for LDEs on the 28-MHz amateur band each evening from 18 to 27 December 1989. On the evening of 20 December at 20:05 eastern standard time I heard and recorded an extraordinary example of a sextuple LDE. The multiple echoes, resulting from a single-sideband (SSB) transmission, were weak but distinct.

The transmitted signal (about 1 kW into a 5-element Yagi-Uda antenna oriented west-northwest) was the word “tango”. Weak but distinct reproductions of my voice can be heard at 2.9, 4.5, 5.5, 7.1, 8.1 and 9.5 s. I timed the echoes by repeatedly playing back the tape into a storage oscilloscope and manually marking the trace of a particular echo. I estimate the uncertainties in these times to be of the order of 0.1 s. The first and fourth echoes are harder



a, Unfiltered receiver output consisting of transmitted single-sideband signal “tango” and noise. Syllables “tan” and “go” are marked by the arrows *t* and *g*, respectively. b, Filtered receiver output. Times at which the syllables “tan” and “go” can be heard on the tape recording are marked approximately by the arrows *t* and *g*, respectively. See text for details. Duration of the oscilloscope trace is 10 s in each photograph. Copies of the magnetic tape cassette recording available on request from A.K.G.

to hear than the others: the second seems to be Doppler-shifted up very slightly in frequency. The receiver output (*a* in the figure) has a bandwidth of about 2.5 kHz and so the echoes are concealed in the noise. To enhance the echoes, I played the tape-recordings through a narrow-band audio filter, about 150-Hz wide, at about 900 Hz (*b* in the figure). This presentation is not well suited for displaying these weak echoes but, in the case of the stronger echoes, the syllables “tan” and “go” can be heard at the times indicated.

I am certain that this example of an LDE is not an artefact. I had previously used the magnetic tape cassette to record Morse code but not SSB signals, so the echoes are not signals previously recorded and incompletely erased. The transfer of a strong audio signal to an adjacent layer of magnetic tape is a recognized problem but a weak overprint is, because of the geometry of the tape cassette, never subsequently heard on the tape more than 1.5–3 s before or after the strong transmitted signal. I cannot exclude the possibility that the echo at 2.9 s is an overprint, but no echoes are heard on the tape after the next two transmitted words “lima” and “echo”.

Any LDE may be a hoax by which a radio operator transmits a signal, but in this case I had initially adjusted my transmitter at high power at one frequency and then changed to a new operating frequency several kilohertz away. The word “tango” was my first SSB transmission of the evening and it is extremely unlikely that anyone would have so quickly found my new frequency, recorded my signal and re-transmitted it six times over.

The intervals between successive echoes are 1.6, 1.0, 1.6, 1.0 and 1.4 s. Because the echoes are too weak for me to time accurately, I cannot vouch for a perfect regularity in the delay times, but my previous observations of LDEs suggest that some sort of periodic structure exists in LDE delay times^{6,7}.

I believe that LDEs are relatively common, and that a systematic search at high power may provide sufficient data to discover how radio waves can persist essentially undistorted for such long periods of time.

A. K. GOODACRE

Amateur Radio Station VE3HX,
1286 Woodside Drive,
Ottawa, Ontario K2C 2G9,
Canada

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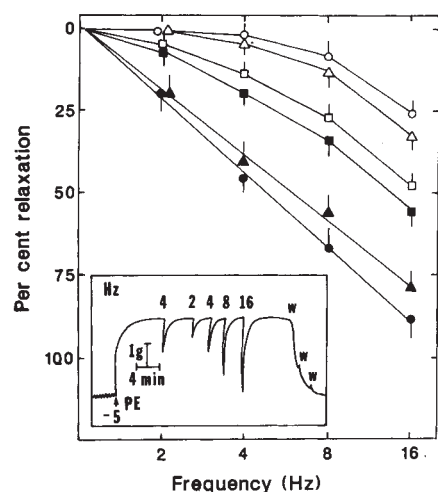
Neurotransmitter identity doubt

SIR—In a recent Letter¹, Bult *et al.* showed that stimulation by an electric field of the isolated and perfused canine ileocolonic junction pretreated with adrenergic and cholinergic blocking agents results in the release of nitric oxide. The authors concluded that nitric oxide is released from the nonadrenergic–noncholinergic neurons innervating this tissue and, therefore, that nitric oxide is the inhibitory transmitter for these neurons. An alternative explanation entirely consistent with these data is that stimulation of these neurons causes release of an unknown neurotransmitter that, in turn, stimulates the underlying smooth muscle to generate nitric oxide.

We find that stimulation of isolated strips of rabbit corpus cavernosum pretreated with guanethidine and atropine promotes the endogenous formation and release of NO, nitrite and cyclic GMP. Formation of these substances in tissues is frequency-dependent, associated with frequency-dependent relaxation of corporal smooth muscle, and all responses are abolished by tetrodotoxin and unaffected by indomethacin. We found that stimulation-induced relaxation of corporal smooth muscle is markedly inhibited by *N*^G-nitro-L-arginine, an inhibitor of the biosynthesis of NO from L-arginine², and by haemoglobin and methylene blue, agents that are known to interfere with the biological actions of NO (refs 3, 4; see figure).

The inhibitory effects of *N*^G-nitro-L-arginine, *N*^G-amino-L-arginine and *N*^G-methyl-L-arginine were nearly completely reversed by addition of excess L-arginine but not D-arginine. These observations suggest that these *N*^G-substituted analogues of L-arginine inhibit formation of NO from endogenous L-arginine. Stimulation-induced corporal smooth-muscle relaxation is accompanied by six- to eight-fold increases in tissue levels of nitrite, the spontaneous oxidation product of NO (ref. 5) and cyclic GMP, the intracellular mediator of smooth muscle relaxation caused by NO (ref. 6). Both of these responses are nearly abolished by the application of 10 μM *N*^G-nitro-L-arginine.

Because penile erection is the result of neurogenetically stimulated relaxation of corpus cavernosum smooth muscle followed by venous occlusion and engorgement of corporal sinusoids with blood, these observations, suggest that penile erection is mediated by NO generated in response to nonadrenergic–adrenergic neurotransmission. Although we have found that stimulation-induced corporal smooth muscle relaxation occurs independently of vascular endothelium,



Effects of inhibitors of NO formation or action on electric-field-stimulation-induced relaxation of corpus cavernosum smooth muscle. Strips of rabbit corpus cavernosum were mounted and equilibrated under 2 g tension for 60 min in oxygenated Krebs-bicarbonate solution, and subjected to 10-V, 0.2-ms square-wave pulses at the indicated frequencies for 10 s. Bathing media contained 5 μ M guanethidine and 1 μ M atropine. Inset, a typical tracing illustrating relaxation of phenylephrine (PE; 10 μ M)-precontracted strips. W, washing of strips. Filled circles, responses to stimulation in the absence of additions; open circles, presence of 30 μ M N^2 -nitro-L-arginine; open triangles, presence of 30 μ M N^2 -nitro-L-arginine plus 300 μ M L-arginine; filled triangles, presence of 30 μ M N^2 -nitro-L-arginine plus 300 μ M L-arginine plus 10 μ M haemoglobin; open squares, presence of 10 μ M haemoglobin; filled squares, presence of 10 μ M methylene blue. Data are means $\pm$ s.e.m. of 12–18 strips from 3–5 rabbits. Experimental values are significantly different ($P < 0.01$) from corresponding control (filled circles). (Experimental details available from L. J. I. on request.)

whereas relaxation elicited by acetylcholine is endothelium-dependent, we cannot conclude unequivocally that NO is released primarily from the neurons. The data equally support the alternative conclusion that an unidentified neurotransmitter stimulates the corporal smooth muscle to generate NO. The question of whether NO is the neurotransmitter remains unanswered.

L. J. IGNARRO
P. A. BUSH
G. M. BUGA

Department of Pharmacology and
Division of Urology,

J. RAJFER

Department of Surgery,
University of California,
Los Angeles,
California 90024, USA

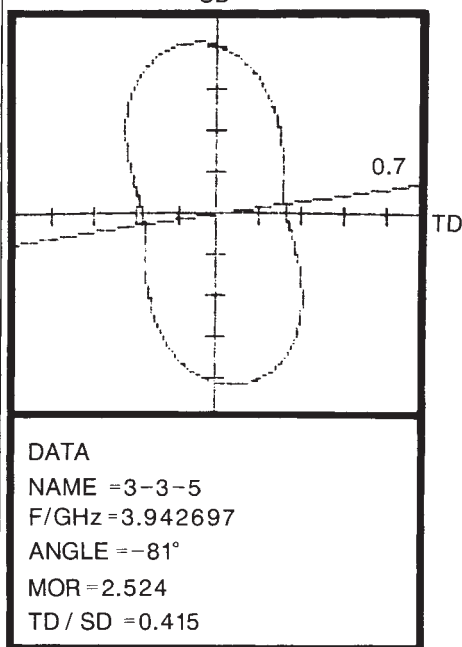
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Orientation test

SIR—Determination of the orientation of fibrous components in biological tissues is essential for the study of the relationship between fibre structure and physical properties of the tissue. Conventional methods such as mechanical breaking strength and X-ray diffraction are not always convenient for practical use as a long time is required for determining fibre orientation. I have devised a method for determining the fibre or molecular orientation of polymer films, paper and blood vessels in as short a time as 30 seconds by using polarized microwaves^{1–5}. I have now applied my technique to cow leather to determine the orientation and the distribution of the fibre. The new microwave method would be applicable to the determination of fibre orientation in various biological tissues such as deer and pig skin or human blood vessels.

The figure shows the angular dependence of transmitted microwave intensity for a sample of cow leather. The angular dependence gives an orientation pattern like an egg cocoon, reflecting the preferred orientation of fibres in the sheet plane. The direction of the minimum transmitted microwave corresponding to the direction of the main chains of fibres deviates by 81° from the standard direction.

The direction of maximum mechanical breaking strength deviates by about 80° from the standard direction and approximately coincides with that of the minimum transmitted microwaves. Electron microphotographs show that the main chains of collagen fibres constituting cow leather



Angular dependence of transmitted microwave intensity at 3.9 gigahertz for a cow leather with a sample size of 100 (length) $\times$ 100 (width) $\times$ 1.17 mm (thickness) and a weight of 860 gm⁻². SD, standard direction; TD, transverse direction; MOR, ratio of maximum to minimum intensity (anisotropy).

are orientated mainly in the direction of maximum mechanical breaking strength.

The orientation patterns showing a fairly strong anisotropy for the cow leather varied remarkably with changing position, indicating that the orientation of fibres in cow leather is not uniform. The orientation angle and anisotropy also change with changing position, implying that the main-chain direction of the collagen fibres and the degree of orientation vary with position. The observed change in anisotropy with varying position may be related to the function of the skin. For example, expansion and contraction corresponding to the movement of the cow's body may require the preferable orientation of collagen fibres in a fixed direction.

SHIGEYOSHI OSAKI

Nichii Co. Ltd,
2-2-9, Awajimachi,
Chuo-ku,
Osaka 541, Japan

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High-temperature granulites

SIR—Hayob *et al.*¹ describe exsolved high-temperature ternary feldspars from a granulite xenolith, which they claim to have been produced in a regional-scale deep crustal metamorphism “no more than 30 Myr ago”. They also claim that these xenoliths record minimum metamorphic temperatures that are “the highest yet known to be preserved in deep seated metamorphic rocks”. Although the discovery of such high-temperature granulites is important in the context of the continuing debate on the origins of granulites², Hayob *et al.* fail to justify their claims for the relative youth of the high-temperature metamorphism and ignore some published literature documenting outcropping granulites, which from various petrological criteria can be shown to have been metamorphosed at temperatures greater than 950 °C. Here I summarize data from one such terrain: the Archaean (~ 3,000 Myr; ref. 3) Napier complex of Enderby Land, Antarctica.

Hayob *et al.*'s temperature estimates come from reintegrated mesoperthite compositions containing 20–23 mol% anorthite. Similar mesoperthites have previously been reported in metapelites (and orthogneisses) from the Napier complex^{4–6}, and are not unique to the occurrence cited by Hayob *et al.* That these data have not been referred to by Hayob *et al.* is a serious omission, particularly given the independent mineral assemblage and thermometric evidence in

the Napier complex for temperatures in the range 980–1,020 °C on a regional (> 5,000 km²) scale^{5–10}. Thus, the xenoliths described by Hayob *et al.* are neither unique nor new in their conditions of formation.

Hayob *et al.* also provide no convincing arguments for the young age of the granulite metamorphism recorded in their xenoliths; the ideal proof would have been zircon or monazite U–Pb dating. Hayob *et al.* cite a lack of retrogression and the inference of 900 °C temperatures from exsolved feldspar lamellae as evidence for recent metamorphism followed by sampling from a young, hot crust (page 267 of their paper). These arguments are tenuous, as factors other than time (for example, the access of fluids and the responsiveness of the mineral assemblage to changes in pressure or temperature) may control the extent of retrogression and re-equilibration.

With respect to retrogression and preservation of assemblages, an important aspect of the Napier complex granulites is that they cooled isobarically and stayed buried in the deep crust for at least 500 Myr and apparently for up to 2,000 Myr following the Archaean high-temperature metamorphism^{3,9}. Despite this prolonged residence in the lower crust, pristine, little-retrogressed high-temperature assemblages such as sapphirine–quartz, orthopyroxene–sillimanite, sillimanite and calcic mesoperthites similar to those described by Hayob *et al.* in composition and appearance (see for example, Fig. 8 of ref. 3; Fig. 2a of ref. 11) occur throughout the Napier complex.

Thus, the textures described by Hayob *et al.* can also be found in exposed Archaean granulites which spent much of their post-Archaean history buried at least 20 km deep in the crust⁹. The textures themselves do not imply a recent metamorphism, and caution is needed to construct a syn-eruptive 'palaeogeotherm' from such crustal xenoliths. In Enderby Land, the Napier complex granulites would have been available for sampling by passing magmas during a considerable time interval. In fact, basaltic magmatism and dyking did occur in Enderby Land in the late Proterozoic³, with little impact on the enclosing dry granulites.

The ages of the xenoliths described by Hayob *et al.* remain to be established. It would be exciting if young metamorphic ages are indeed produced using reliable techniques. The alternative, that the young magmatism is sampling unrelated and possibly much older buried granulite regions, well removed from the source of the magmatism, is equally if not more plausible.

SIMON L. HARLEY

Department of Geology and Geophysics,
University of Edinburgh,
Edinburgh EH9 3JW, UK

SIR—Hayob *et al.*¹ claim to demonstrate the formation of young high-temperature granulite-facies metamorphic rocks in the lower crust as a result of underplating by basaltic magma. Although we would not deny the possibility of recent or present-day basaltic underplating and granulite-facies metamorphism, we feel that these authors have failed to demonstrate its occurrence. Hayob *et al.*'s arguments revolve around their finding of high-temperature exsolved feldspar compositions in granulite-facies xenoliths, which have been erupted with basaltic tuffs by Quaternary volcanism in central Mexico. These feldspars (mesoperthites) yield temperature estimates of 800–950 °C for formation of the existing lamellae and host compositions, and temperature estimates of 950–1,125 °C for formation of the integrated compositions of the original feldspars. Hayob *et al.* claim that these temperature estimates set a series of precedents, and make several misleading statements, such as:

mesoperthites yield minimum reintegrated temperatures (950–1,125 °C) that are considerably higher than those typically obtained from exposed granulites (p. 266). The mesoperthites provide evidence for regional metamorphism at $T > 950$ –1,125 °C, higher than any temperatures recorded previously in regionally metamorphosed rocks of undoubted sedimentary parentage (p. 267).

But there are at least two well-known terrains where both the conditions of metamorphism and mesoperthites similar to those described by Hayob *et al.* have been documented: the Lewisian complex of Scotland and the Napier complex of Antarctica; in both cases the relevant rocks are exposed on the surface, forming parts of regional granulite-facies terrains of Archaean age.

In the Lewisian case, the integrated compositions of mesoperthite grains, which coexist with antiperthite grains, suggest temperatures above 1,000 °C^{12–14}. Although they occur in a granitic rock for which the proportion of igneous and metamorphic features has been debated^{12,14,15} they have clearly survived a very long cooling and subsequent history before being exhumed at the surface^{14–16}. Other nearby Lewisian rocks, including meta-sedimentary ironstones, provide evidence of temperatures of $\geq 1,000$ °C^{14,17}. The Napier complex in Enderby Land includes mesoperthites and other minerals and assemblages in metasediments, which provide clear evidence of high-temperature metamorphism exceeding 900 °C^{5,6,9}. This Archaean complex probably extends for >15,000 km² and has a long exhumation history^{9,18}.

The Lewisian and Napier rocks demonstrate that granulite-facies rocks with evidence of metamorphic temperatures of greater than 900 °C and mesoperthites can survive cooling to surface conditions.

Although the xenolith assemblages of Hayob *et al.* may indeed never have equilibrated at temperatures below 900 °C, the authors are wrong in implying that this is because the xenoliths never experienced temperatures below 900 °C, other than when they were rapidly quenched by igneous eruption.

Hayob *et al.* assume that their xenoliths have been plucked from the base of the crust at conditions similar to the lowest pressures and temperatures (P and T) preserved in the xenoliths. Such assumptions have been questioned for xenoliths of granulite-facies rocks and even for some mantle-derived xenoliths, because the P – T conditions may have been frozen into the rocks long before eruption^{19,20}. The Lewisian and Napier rocks demonstrate that the P – T conditions of the Hayob *et al.* xenoliths could be preserved from as long ago as the Archaean. Thus Hayob *et al.*'s assumption that their xenoliths are a product of Tertiary basaltic underplating is unwarranted; from the evidence provided, the xenoliths could have been plucked from a metamorphic complex similar to the Lewisian or Napier complex, lying at any depth in the central Mexican crust and of any age. To demonstrate very high-temperature granulite-facies metamorphism by Tertiary basaltic underplating, Hayob *et al.* need to provide direct, unequivocal evidence of the age of the relevant mineral equilibria in their xenoliths.

BEN HARTE

Department of Geology and Geophysics,
University of Edinburgh,
Edinburgh EH9 3JW, UK

ANDREW BARNICOAT

Department of Earth Sciences,
University of Leeds,
Leeds LS2 9JT, UK

HAYOB *ET AL.* REPLY — Harley and Harte and Barnicoat question our inference that granulite-facies paragneiss xenoliths from Quaternary eruptions in central Mexico preserve the highest regional metamorphic temperatures yet recorded in deep-seated crustal rocks. Elsewhere, Harley² has compiled P – T information for more than 90 granulite terrains, only five of which record temperatures in the range 950–1,000 °C. By contrast, six of the paragneiss xenoliths from central Mexico record minimum temperatures in the range, 1,050–1,125 °C. Thus, the Mexican paragneisses record temperatures at least 100 °C higher than previously reported for regionally metamorphosed rocks; we maintain that these xenoliths do indeed record the highest regional metamorphic temperatures yet cited for crustal granulites. Our statement¹ that "the mesoperthites yield minimum reintegrated temperatures that are considerably higher than those typically [italics added] obtained from exposed granulites" is also

TEMPERATURES RECORDED BY EXSOLVED FELDSPARS

Granulite terrains	Ref.	Xenolith localities	Ref.
Lewisian, Scotland*			
400–420	12	El Toro, Central Mexico	
400–560	21	870–910	1
580	14		
		La Joya Honda, Central Mexico	
Adirondacks, USA†		890–960	1
300–500	22		
		Los Palau, Central Mexico	
McCullough, USA		930–960	1
600	24		
		La Olivina, North Mexico	
Napier, Antarctica		840–890	29
420	11		
630	5	Kilbourne Hole, USA	
		840–890	30
		890–920‡	30
Oaxaca, Mexico*			
420–470	25	Baidlandhill, Scotland	
630–710‡	25	760	31
		Partan Craig, Scotland	
Laramie		840‡	31
Anorthosite, USA§			
560	26	Hoggar, Algeria	
		740–840‡	32
Wilson Lake, Canada			
700–740‡	27		

Temperatures (°C) calculated at 1.0 gigapascals with the ternary-feldspar model of ref. 23.

*These feldspars may be of igneous origin.

†Temperatures were not recalculated as no exsolved feldspar data were given.

‡Calculated temperatures for the three components²³ do not agree within ± 50 °C.

§Equilibration temperatures from a slowly cooled igneous suite are shown for comparison, calculated at a pressure of 0.5 gigapascals.

|| Temperatures are based on coexisting antiperthite and perthite, and probably represent peak metamorphic conditions.

valid. Harley² shows that most granulites record temperatures of 850 °C or lower.

The exsolved and the reintegrated mesoperthites from the Mexican xenoliths are unique in their ternary nature. Harley and Harte and Barnicoat cite several occurrences of mesoperthites in metapelites from the Archaean Lewisian and Napier complexes, which they believe are similar to those found in the Mexican xenoliths. Mesoperthites from the Lewisian complex occur in discordant granite sheets^{12,21} and are probably igneous^{14,21}, rather than metamorphic, in origin. Moreover, a key issue of our paper was the significance of the ternary nature of the exsolved feldspar compositions, a point that Harley and Harte and Barnicoat have apparently misunderstood. As a result of the high temperatures typically achieved during granulite-facies metamorphism, alkali feldspar from exposed granulites exsolve on slow cooling to low temperatures²².

By contrast, the failure of the host and lamellae feldspar in the Mexican paragneiss xenoliths to re-equilibrate below ~ 800 – 950 °C strongly suggests that the samples were not subjected to slow cooling below these temperatures. Although Harley and Harte and Barnicoat reject this argument, our conclusions are supported by many mineralogical and petrological data on natural samples from both outcropping granulite terrains and granu-

lite xenoliths. We have recalculated temperatures²³ recorded by exsolved feldspar occurring in exposed granulites and xenoliths (see table). Feldspars that have experienced slow cooling to low temperatures (for example, in the Napier and Lewisian complexes) are invariably chemically (and structurally) reset to temperatures in the range 400–650 °C^{5,11,12,14,21,22,24–26} and have not survived long exhumation histories in an unaltered form. One possible exception is the Wilson Lake complex in Labrador²⁷, where coexisting feldspars may record temperatures in the range 700–740 °C (see table). Calculated temperatures from this locality are extremely discordant, however, and the host-lamellae pairs cannot represent equilibrium assemblages²³.

Although extremely ternary feldspar compositions are plotted by Harley⁶ on a ternary diagram for Enderby Land granulites, he gives no feldspar analyses. So it is impossible to evaluate the equilibration temperatures of coexisting host and lamellae feldspars. Using a graphical ternary feldspar thermometer²⁸, several of the reintegrated compositions and one exsolved(?) alkali feldspar indicate unreasonably high temperatures, in excess of 1,200 °C. Harley⁶ concedes that his defocused-beam analyses of mesoperthites are “indicative only”, but the precise meaning of this statement is not clear. We interpret it to

suggest that the reintegrations are not representative of the true compositions of the original homogeneous feldspars. Thus, we know of no mesoperthites in exposed granulites that preserve high-temperature ($> 7,700$ °C), equilibrium exsolutions. By contrast, coexisting exsolved feldspars found in xenoliths sometimes preserve more ternary compositions and record higher temperatures of last equilibration^{1,29–32} (see table) because they have not been subjected to slow cooling at lower temperatures.

Harley and Harte and Barnicoat also question the youth of the granulite metamorphism but, in addition to the ternary feldspar compositions, geological constraints and heat flow measurements also imply a relatively young age for the last thermal event of the Mexican plateau. Mexico has been the locus of extensive Tertiary volcanism and uplift. The Tertiary Sierra Madre Occidental ignimbrite province, just west of the paragneiss xenolith localities, comprises the largest volume of erupted material exposed on Earth. These processes must have imposed a major thermal imprint on the lower crust of Mexico during the Tertiary. The central Mexican plateau is a region of high heat flow; the geothermal gradients of 20–28 °C km⁻¹ estimated in our paper¹ are in agreement with gradients predicted from heat flow and crustal thickness measurements about 100 km to the north (cited in ref. 1). Although it is difficult to extrapolate gradients obtained from near-surface heat flow measurements to deeper levels, present-day heat flow measurements clearly indicate a relatively young thermal event.

J. L. HAYOB

E. J. ESSENE

Department of Geological Sciences,
University of Michigan,
Ann Arbor, Michigan 48109, USA

J. RUIZ

Department of Geosciences,
University of Arizona,
Tucson, Arizona 85721, USA

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A tale of two cultures

Robert Kuttner

America's Struggle for Leadership in Technology. By Jean-Claude Derian. MIT: 1990. Pp 309. £26.95, \$29.95.

JEAN-Claude Derian is in the distinguished tradition of Tocqueville — a French visitor who grasps US institutions better than most natives. Derian wrote this succinct book while he was science counsellor at the French Embassy in Washington during the mid-1980s. His subjects are the confused objectives of US technology policy, and its mixed effect on the competitive standing of industry. In part, the book is an efficient tour of the usual horizon: the role of NASA, the Pentagon and such “big science” projects as the Strategic Defence Initiative (Star Wars) in incubating and channelling new technologies; the distinct subculture of Silicon valley; a comparison of technology policy in the United States, Japan and France, and so on. This, of course, is well-worn territory, yet Derian manages to work it better than most.

But Derian's original contribution, which makes the book especially worth reading, is his analysis of the two cultures of high-technology industry — a ‘sheltered’ culture of large companies either with close linkages to government or dwelling within regulated or cartelized industries, and which compete mainly on the basis of technical advance; and an entrepreneurial ‘exposed’ culture of companies and industries competing openly (and fiercely) on the basis of price. “The sheltered culture”, writes Derian, “has resulted from what seems to be an accident of history: the involvement of the federal government in the generation of advanced military technology in the aftermath of World War II.” Most military contractors fit into this category, though regulation can be as efficient a basis for shelter as government procurement.

AT&T was perhaps the epitome of the US sheltered company. Although privately owned, it was a regulated monopoly. It could divert earnings to Bell Labs, the crown jewel of industrial laboratories, and pass the cost on to its captive customers. AT&T was superb at big technical innovations like digital switching, which its captive regional telephone companies were compelled to buy and which drove down the cost of basic phone calls and expanded its market. But as computing and telecommunications converged into a single digital technology, AT&T's sheltered culture made it very slow to compete for the new markets in the thousands of devices that could fit on the end of a telephone wire. These devices are highly price competitive, and successful purveyors are

necessarily entrepreneurial. After deregulation, AT&T fell on its face attempting to sell personal computers, though it has been highly successful marketing its UNIX system, the fruit of Bell Labs.

IBM, by contrast, is the quintessential ‘exposed’ company — entrepreneurial, market driven, and innovative in a different fashion. “Big Blue”, despite its market power, sides with supporters of open systems, allowing its machines both to be cloned and to remain the industry standard, as a strategy for keeping itself on its toes.

Although defence firms tend to epitomize the sheltered firms, the correlation is far from perfect. Some companies, Boeing for example, began as almost exclusively suppliers to the US Air Force, yet made the transition to commercial markets while still drawing on the benefits of close research and procurement links to government, and the oligopolistic character of the aircraft industry.

What is refreshing about Derian's version of this analysis is his catholicity. In an era of government-bashers versus mercantilists, he is in neither category. His message is not that government involvement is a millstone — or that it is a salvation. Nor does he discern a simple correlation between government linkage and entrepreneurial failure. His point is that one must get down to cases, which he does brilliantly. In the heyday of

Dwight Eisenhower's “military-industrial complex”, the linkage of sheltered firms to the Pentagon and NASA produced great technical advance, and commercial benefit for big US companies, but this era is over. Derian nominally classifies Japanese industry as mostly exposed rather than sheltered, but in reality (as he seems to agree), what Japan really manages is a more competitive blend of the two cultures. Firms are partners in interlocked industrial groups: the state subsidizes targeted breakthroughs frankly aimed at commercial advantage; it relies on cartelization and market closure when that serves its purposes, yet its companies are fiercely price-competitive in world markets. Japan seems sheltered *vis a vis* imports, but highly open in export markets.

America's problem today is that it no longer has the military basis for incubating commercial technologies, whereas its 40-year reliance on shelter has sapped the entrepreneurial vitality of its firms that must compete in an increasingly exposed global culture. The country is also hobbled by peculiar ideological blinders. So long as the ostensible goal is basic research or military supremacy, the prevailing ideology allows government subsidy of technology. But in a nation that believes deeply in Adam Smith and David Ricardo, deliberately using government to help create commercial advantage is considered sinful. Aid to the semiconductor industry could be channelled only through the Pentagon, disdaining commercial spillovers. Aid to high-definition television (HDTV) was rejected by the Bush White House as a form of the loathed industrial policy. Neither Japan nor France nor the EC commission has this Anglo-Saxon neurosis (which is currently



Mountain melody — Nepalese monks blow their long brass horns, producing “a low primitive sound that rolled across the valley to the mountains”. Mike Harding, British entertainer and keen conservationist, trekked through the Himalayas of India and Nepal while making a film about the effects of deforestation. The photographs he took together with his affectionately written account of the journey combine to provide a superb portrayal of this breathtakingly beautiful corner of the world. Published by Viking, price £16.95, \$29.95.

shared only with Mrs Thatcher). As a result, the United States ends up with a less efficient blend of the exposed and sheltered cultures than most of its trading partners.

Nearly all of this book is about the United States, though in a closing chapter Derian makes it clear that he favours both a more entrepreneurial, less sheltered European industry, particularly as the single market approaches, but also the benefits of efficient shelter such as the commission's technology research consortia (ESPRIT *et al.*). Europe has companies clearly in the exposed, entrepreneurial tradition (Philips, Olivetti), and it needs more. As a Frenchman, Derian possesses both an appropriate disdain for *etatisme* and an appreciation for the benefits of a soupçon of *dirigisme* if used in moderate amounts. "The omnipresence of the state and the state-company symbiosis, are at once sources of strength and weakness in France", he notes.

Like other European visitors to the United States, Derian occasionally throws up his hands in gallic bewilderment at the sheer perversity of much of the country's technology policy and its tendency to squander its remarkable legacy for the sake of geo-political and ideological goals. An American observer of science, military and economic policy must sadly concur with Derian's analysis. The book, incidentally, was originally written in French. The translation, by Severin Schaeffer, is a superb rendering in idiomatic and technically accurate English. □

Robert Kuttner writes for *The New Republic* and *Business Week* and is at 61 Dean Road, Brookline, Massachusetts 02146, USA.

New in paperback

■ Three new additions to Oxford's Microscopy Handbook series are *Introduction to Crystallography* by C. Hammond (£9.95), *The Operation of Transmission and Scanning Electron Microscopes* by Dawn Chescoe and Peter Goodhew (£7.95) and *Cryopreparation of Thin Biological Specimens for Electron Microscopy* by Norbert Roos and John Morgan (£9.95).

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■ *Retroviral Proteases: Maturation and Morphogenesis* is a collection of articles on the structure and function of these molecules, including HIV-1. Edited by Laurence Pearl and published by Macmillan, price is £27.50. □

Rich material

B. F. Schutz

A Journey into Gravity and Spacetime By John Archibald Wheeler W. H. Freeman: 1990. Pp 257. £16.95, \$32.95.

THE distinguished physicist John Archibald Wheeler's long-awaited 'popular' book on general relativity has finally appeared, and it is a tour-de-force. Aiming to give intelligent non-physicists the opportunity to gain real insight into gravity, Wheeler has written a gem of a book that will be required reading for the experts as well. For where most such books skirt around the parts of the theory that seem mathematically the most abstract, Wheeler tackles them head-on. He repeatedly finds beautifully lucid ways into the heart of the subject, using only a minimum of algebra and geometry. Helped by many high-quality illustrations, his fluent writing makes it all seem easy. The book demonstrates yet again Wheeler's life-long message to physicists: what is deep is also simple.

Wheeler is known as both a creative scientist and a teacher. As Richard Feynman's mentor, he contributed enormously to the foundations of quantum electrodynamics. As one of the first 'mainstream' physicists to move into general relativity, he fathered a school of research from which has sprung some of the most important work on black holes, gravitational waves and cosmology — indeed, he coined the very name 'black hole'. His introductory textbooks on special relativity (with Edwin Taylor) and general relativity (with Charles Misner and Kip Thorne) have been very influential, and are widely used in university courses.

Wheeler's writings have a characteristic style, which is not to everyone's taste. He makes extensive use of analogies and of asides that explore byways along the main road of exposition. In the present example, I feel that this approach works very well. The asides, presented in text on a shaded background, typically present material that needs extra mathematics. The analogies are unusually well constructed: no mere similes, they usually give considerable insight into the point at hand.

Nothing better illustrates Wheeler's ambitious approach than his treatment of space-time curvature. Experts know that the components of the curvature tensor outside a black hole all have roughly equal magnitudes, but differ by signs and factors of two. This might not seem a point worth addressing in a 'popular' exposition, but for Wheeler it is absolutely central. The components have the relationships that they have to one another in order that the rule he has previously given us for the curvature of space-time — what the experts

call Einstein's field equations but what in this book become a simple word-equation relating two-dimensional notions of curvature to the energy and momentum in space-time — should hold in spherical symmetry. Wheeler makes his point without ever needing the language of tensor analysis.

In a similarly simple and direct manner, Wheeler makes the principle of maximum proper time the foundation of his discussions of motion in space-time. Most popular accounts would, I suspect, refer to this only in passing, or as a consequence of other considerations. Wheeler's method is for him the simplest way into the subject, allowing him a straightforward generalization from the law of motion in flat space-time, and permitting him to explain planetary motion in the same breath as the twin paradox.

There are equally masterly discussions of gravitational waves — why they are transverse and quadrupolar — of the Penrose process, and of many other features of space-time and gravity that are usually considered too difficult to explain in simple ways. There are, of course, parts of the book I could criticize. Wheeler's style does sometimes confuse at least temporarily, such as the occasions on which he states an important result with no justification and neglects to mention that he will prove it in the next section or chapter. But shortcomings like this did nothing to inhibit the feeling that grew in me as I read through the book, that this is a masterpiece of explanation.

A reader with no background in general relativity who takes the time to think about Wheeler's arguments and explanations should find this the most satisfying of expositions on general relativity; to come away having understood and even (for the spherical case) essentially to have solved Einstein's field equations should more than reward the effort invested. For those who think of ourselves as expert in the theory, the book shows us that we still can learn things from the old master. And for those of us who also teach the subject, there is rich material here for our lectures. I think the book would do very well as auxiliary reading in a lecture course to supplement one of the standard textbooks, because it fleshes out with words and explanations the abstractions of the equations. This is a book that should therefore be in every academic library as well as in quite a few personal ones. □

B. F. Schutz is in the Department of Physics, University of Wales College of Cardiff, PO Box 913, Cardiff CF1 3TH, UK.

■ Just published by Oxford is *General Relativity*, in which I. R. Kenyon reviews the field, including the general and special theories, black holes, cosmology and the search for a quantum theory of gravity. Price is £30 (hbk), £15 (pbk). □

Sugared pill

Leslie Crombie

Comprehensive Medicinal Chemistry. The Rational Design, Mechanistic Study and Therapeutic Application of Chemical Compounds. Editor-in-chief Corwin Hansch. Six volumes. Pergamon: 1990. Pp. 5,494. £1,145, \$1,995.

UNTIL the development of synthetic organic chemistry in the latter part of the past century, our stock of medicinal chemicals came largely from plant sources, usually used impure in the form of herbal preparations. Such sources provided drugs such as morphine, codeine, quinine, cocaine, ergot, curare, ephedrine, reserpine, salicin, digitoxin and, from microbes, many antibiotics. Progress in synthesis put at the disposal of the medicinal chemist an enormous range of structures, together with the means for modulating their design. It is of course one thing to have available lots of chemicals, and quite another to select useful medicinal compounds from among them. In the early days, this process was often a matter of guesswork and luck. Naphthalene, thought to be useful as an internal disinfectant, was tried for the eradication of worms, for example. Fortunately, a dispensing error was made and acetanilide was administered to a patient suffering from a fever as well as from worms. The drug abated the fever, and the value of the closely related phenacetin and the much used paracetamol is derived from this observation. It was noticed that the anti-syphilitic mercurial drug merbaphen produces a striking increase in urine flow; this observation led to the use of diuretics to treat congestive heart failure.

Although sharp observation is as important as it ever was, the design and evolution of medicinal compounds is now a highly sophisticated business involving enormous expenditure. It is also highly interdisciplinary, covering chemistry, biochemistry, physics and many areas of biology and pharmacy, as well as requiring knowledge of patents, regulations and similar topics. This complex amalgam of interests directed towards the specific aim of producing new and better medicinals is the theme of the six volumes entitled *Comprehensive Medicinal Chemistry*, which describe common ground for those engaged in this endeavour. Despite the massive nature of the work (as well as the vast number of pages there are more than 230 authors and editors from 15 countries) organic synthesis, which occupies much of a medicinal chemist's time, is not discussed. But this omission is reasonable as organic synthesis is a large topic with well-established sources of information of its own. Starting with a candidate drug

structure, rather than discussing how it may be made, also makes the work more accessible to those in other disciplines.

In many ways the second and third volumes provide the core information on new drug discovery. Volume 2, *Enzymes and Other Molecular Targets*, covers agents acting on oxygenases, electron-transport systems and pyridoxal-dependent systems, on metabolic processes, on hydrolases and peptidases, on cell walls and on nucleic acids. Volume 3, the largest of the set, deals with membranes and receptors, covering neurotransmitters, peptidergic and intracellular receptors as well as drugs acting on ion channels and membranes. These are excellent comprehensive volumes.



Healing plant — *Trachelospermum jasminoides* or Chinese star jasmine. Traditional uses include treatment of rheumatic pains, muscular spasm and injuries. This is one of the 150 most commonly used plants in traditional Chinese medicine catalogued in *Medicinal Plants in China*. Publisher is the World Health Organization, price is \$40 (pbk). *Plants for Medicines: A Chemical and Pharmacological Survey of Plants in the Australian Region* gives details of the phytochemical testing of nearly 2,000 species of Australian flora. Published by CSIRO, price is \$70.

Quantitative structure-activity relationships are considered in volume 4. The idea of factorizing drug activity into physical contributions (hydrophobic, electronic, steric and so on) has been central to the Hansch approach and has shown how important hydrophobicity is to drug transport. Application of structure-activity data assists the optimization of 'lead' compounds to form effective drugs with economy of effort by aiding the selection of the most significant structures for synthesis. The burgeoning subjects of molecular modelling and computer graphics are also covered in this volume. Volume 5 deals with biopharmaceutics — drug delivery, distribution, absorption and related topics. Analytical methods are also covered, although this will be familiar ground to well-trained organic chemists.

Volume 1 seems to me to be of the most general interest. Not only does it give historical perspective, it treats, in rela-

tively uncomplicated form, areas that the medicinal chemist should know something of — human physiology, cell architecture, the immune system, genetic engineering, process development and similar subjects. Socio-economic topics are also considered — government control, toxicological evaluation, clinical testing, patents, trademarks, post-marketing surveillance, sources of information and 'orphan' drugs (drugs that are useful yet, for various reasons, cannot be expected to make any financial profit). There is a good cumulative subject index in volume 6, though there is no author index relating to the many references cited. An unusual feature is the drug compendium — a collection of 5,500 structural formulae of drugs that have been used or studied in man, together with log P and pK_a data.

Although appreciating the constraints involved in producing a work of this nature, I would like more attention to have been paid to stereochemistry, optical resolution and other methods for producing drugs in optically pure forms. Frequently, only one stereoisomer of a drug is active, other isomers merely adding to the body's burden of excretable material. In some cases, the matter is much more serious, thalidomide being a classic example. The R(+) isomer has the desired sleep-inducing effect, but the S(−) is teratogenic. As spatial arrangement is so important in medicinal chemistry, I was surprised that this parameter is not indicated on many natural product formulae illustrated in the otherwise admirable "Chronology of Drug Introductions" in volume 1.

This major work has been well planned, and the high proportion of practising industrial authors (many from the United States and the United Kingdom) has given the result authenticity. Despite its size, the work is really very readable and the organization helps to remedy one's lack of knowledge in some areas. One of the contributors writes, overmodestly, "it is unlikely that anyone will be reading this volume from cover to cover. But in that improbable event..." My riposte is "why not?". This is exciting stuff for an organic chemist keen on medicinal chemistry.

The main barrier to potential readers will be that the volumes will be non-loan, to be read only in the library rather than, as they should be, in the comfort in an armchair at home. A serious pharmaceutical company should consider providing one or more loan sets. Compared with the cost of developing a saleable drug, expenditure would be minute and the return in terms of education of its staff would repay it many times. Sadly, most universities and polytechnics in Britain at least will be lucky to have sight of a non-loan set. □

Leslie Crombie is in the Department of Chemistry, University of Nottingham, Nottingham NG7 2RD, UK.

Ancient paradox

Lawrence Guy Straus

Thoughtful Foragers. By Steven J. Mithen. *Cambridge University Press*: 1990. Pp.289. £35, \$59.50.

IT IS refreshing to read the work of someone with ambitious intellectual goals, someone who not only achieves most of them, but does so with healthy self-doubts and a sense of humour. Mithen tries to bridge the gulf between materialist processual archaeology and the self-proclaimed radical post-processual variety. In this dissertation-turned-book, he seeks to explain why Mesolithic hunters in northern Europe killed specific game animals and why Upper Palaeolithic hunters in southern Europe painted caves. Although the first question may be a bit esoteric (unless you follow the burgeoning literature on hunter-gatherer ecology and optimal-foraging theory), the second is one of the most hotly debated among prehistorians. Mithen's "audacity" in tackling these questions is great: although some of the results of his work are shaky, by and large his suggestions make sense, even if they do not provide the whole answer.

Mithen's argument is an eclectic, usually convincing combination of game theory in the guise of "eco-psychology", ethnographic analogy, game ecology, modified optimal foraging theory, Mesolithic and Upper Palaeolithic archaeology, and computer simulation. The key argument involves the obvious fact that the archaeological record is (partly) the product of decisions made by anonymous, individual humans. His book is an attempt by a practical archaeologist to reconcile

the fact that hunter-gatherers had to eat with the demands of the post-processualists that individuals operate in a world of symbols and social status. Let us not kid ourselves, however; Mithen is looking at statistical patterns. Individual Stone Age hunters as a group still did act rationally in the long run, even if Mithen does acknowledge, refreshingly, that individuals often miscalculated, erred, operated on the basis of imperfect information, and so on — just like their modern counterparts. (In this vein, I found the book more realistic than much of the more abstract literature on optimal foraging.)

One of the paradoxes of *Thoughtful Foragers* (I picture Rodin's "Thinker" seated on a coastal rock, bow on his knee, contemplating a distant stag and a nearby limpet shell) is the argument that Mesolithic hunters in Sweden were engaged in fierce interpersonal rivalry as to who was the greater killer, whereas on the other hand the Upper Palaeolithic people of southern France and northern Spain stressed cooperation. I find the competition aspect of the Mesolithic argument a bit strained, although Mithen obtains a nice fit between his simulated meliorizing model and the faunal assemblages from Swedish sites (in, supposedly, an optimum-foraging environment). I am more dubious about the early Holocene environments along the south German Danube being as poor as Mithen thinks. And many of the Mesolithic faunal assemblages are so small as to make some of the supposed fits highly questionable.

As one of the producers of much of the data Mithen uses for his analyses of Upper Palaeolithic subsistence in Cantabrian Spain, I am gratified that the hypothesis of intensification by means of diversification and specialization I first published in 1977

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is confirmed by Mithen's manipulations. I also agree that much (although not all) of the cave art of the region has to do with the storage and transgenerational transmission of information critical to hunting success under conditions of long-term climatic and prey population instability during the Upper Pleniglacial and Late Glacial. The art (among many other things) undoubtedly does contain cues, mnemonic aids for saga-tellers. Whether this has to do with switching between game drives and stalking, as Mithen thinks, is a bit more problematical. (I could make counter-arguments for aspects of his case, but space prohibits it.) Suffice it to say that I found the arguments stimulating and the use made of our data worthwhile. The text and bibliography suffer from numerous typographical errors (regrettable, given the book's steep price). Mithen seems to have little first-hand Spanish experience; a dose thereof would be good!

Mithen's work should be read and thought about both by armchair theoreticians and by those of us who try to keep producing hard data on chronology, subsistence, technology and settlement as well as on prehistoric social organization and belief systems. □

Lawrence Guy Straus is in the Department of Anthropology, University of New Mexico, Albuquerque, New Mexico 87131, USA.



Father and son — the mummies of Seti I (above) and his son Ramesses II (right). *The World of the Pharaohs: A Complete Guide To Ancient Egypt* by Christine Hobson offers a fascinating glimpse of the lives of both the pharaohs of Egypt themselves, and the explorers who discovered their tombs. Published by Thames and Hudson, price is \$14.95 (pbk).



The ice-core record: climate sensitivity and future greenhouse warming

C. Lorius, J. Jouzel, D. Raynaud, J. Hansen & H. Le Treut

The prediction of future greenhouse-gas-induced warming depends critically on the sensitivity of Earth's climate to increasing atmospheric concentrations of these gases. Data from cores drilled in polar ice sheets show a remarkable correlation between past glacial-interglacial temperature changes and the inferred atmospheric concentration of gases such as carbon dioxide and methane. These and other palaeoclimate data are used to assess the role of greenhouse gases in explaining past global climate change, and the validity of models predicting the effect of increasing concentrations of such gases in the atmosphere.

INFRARED-ABSORBING gases in the Earth's atmosphere raise the mean global surface temperature from -18°C , which it would be in the absence of an atmosphere, to $\sim 15^{\circ}\text{C}$. This trapping of infrared energy emitted by the Earth's surface is known as the greenhouse effect¹⁻⁴. The largest effect is from water vapour, with a significant contribution from carbon dioxide and smaller contributions from ozone, methane, nitrous oxide and, in the past few decades, anthropogenic chlorofluorocarbons (CFCs). The naturally occurring greenhouse effect makes our planet habitable, but there is growing concern about the future of the Earth's climate because of increasing atmospheric concentrations of CO_2 , CH_4 , N_2O and CFCs, which are largely a result of man's activities. This radiative forcing (the change of the planetary radiation balance) is just over 4 W m^{-2} for a doubling of the CO_2 concentration (from, say, 300 to 600 p.p.m.v.). The increase in atmospheric CO_2 , CH_4 , N_2O and CFCs that have occurred since the beginning of the industrial revolution results in a forcing of just over 2 W m^{-2} (ref. 5); thus the anthropogenic greenhouse forcing has already reached a level of about half of the doubled- CO_2 radiative forcing, the canonical large forcing used in theoretical climate studies.

The radiative forcing resulting from doubled atmospheric CO_2 would increase the surface and tropospheric temperature by $\Delta T_e \approx 1.2^{\circ}\text{C}$ if there were no feedbacks in the climate system (see Box 1). But there are many possible feedbacks which may occur in response to an initial forcing. For example, a warming climate is likely to alter the amount of water vapour in the atmosphere, the cloud properties, snow cover and sea ice^{1-4,6}. Doubled- CO_2 sensitivity experiments using the most comprehensive climate models, the three-dimensional general circulation models (GCMs), yield equilibrium warmings between 1.9 and 5.2°C ⁶⁻¹¹. The uncertainty in climate sensitivity obtained from GCMs may, however, be even greater than this. A recent comparison of 14 GCMs showed about a threefold variation in climate sensitivity, attributable mainly to differences in the cloud feedbacks¹². Some simple one-dimensional climate models suggest essentially no net feedback³. Thus the net feedback factor f (the amplification of the no-feedback radiative-equilibrium temperature change) is uncertain in the large range $f \approx 1-4$ (Box 1).

One implication of the uncertainty in climate sensitivity is an even greater uncertainty in the climate response time. The time required for the climate system to approach a new equilibrium temperature after a change of radiative forcing varies at least linearly with f ^{6,13} because the feedbacks come into play in response to the change of temperature, not in response to the climate forcing. A consequence of the large uncertainty in the response time, which may be anywhere from a decade to more than a century, is that the observed global temperature trend of

the past century does not provide a very stringent empirical limit on climate sensitivity; indeed, allowing for the possibility of small unknown climate forcings, such as changes of tropospheric aerosols and solar irradiance, and for unforced climate variability, the temperature trend of the past century does not provide a useful bound on climate sensitivity^{6,14,15}.

A more promising source of empirical information on global climate sensitivity is provided by the changes in Earth's climate during the past few hundred thousand years because the global temperature change was very large, $\sim 4-5^{\circ}\text{C}$ ^{16,17}, the climate change was linked with changes of greenhouse-gas concentration (which by themselves resulted in a direct radiative forcing of $\sim 2\text{ W m}^{-2}$) and the changes were maintained for a sufficiently long time for equilibrium to be achieved, unlike the present greenhouse-gas changes. Based on these considerations there have already been attempts^{6,19} to estimate climate sensitivity from conditions during the Last Glacial Maximum, 18,000 years before present (18 kyr BP).

Box 1 Equilibrium temperature, feedback processes and $2 \times \text{CO}_2$ experiments

Earth's atmosphere is heated by short-wavelength solar radiation and cooled by the emission of long-wavelength radiation to space. The planetary radiative energy budget per unit area, N , can be written as the difference of these terms

$$N = \frac{1-\alpha}{4} S - \epsilon \sigma T_s^4 \quad (1)$$

where S is the solar constant ($1,370\text{ W m}^{-2}$), α the planetary reflectivity or albedo (0.3), ϵ the effective emissivity of the atmosphere, σ the Stefan-Boltzmann constant and T_s the surface temperature⁶⁵. At equilibrium ($N=0$) and with no atmosphere this yields $T_e = 255\text{ K}$. Earth's surface temperature is actually 288 K , primarily because of the presence of the atmosphere and the present greenhouse warming is therefore $\sim 33^{\circ}\text{C}$.

If the atmospheric structure and all other factors remain fixed, the response of the system to an increase of radiative forcing ΔQ would be a change in T_e necessary to restore radiative equilibrium, $\Delta T_e = T_e \Delta Q / (1-\alpha)S$ leading to $\Delta T_e \approx 0.3 \Delta Q$. The radiative forcing for a doubling of the CO_2 concentration is $\Delta Q \approx 4\text{ W m}^{-2}$ corresponding to $\Delta T_e \approx 1.2^{\circ}\text{C}$.

The surface temperature changes, ΔT_s , calculated in recent GCM studies for doubled CO_2 ⁶⁻¹¹ are shown in the table. Here we have adopted the feedback terminology of Hansen *et al.*⁶ in which a net feedback factor f is defined by assuming that the radiative forcing for doubled CO_2 is $\sim 4\text{ W m}^{-2}$ and hence $\Delta T_s \approx 1.2 f$. The feedbacks that have been found to be significant in these models (water vapour, clouds, sea ice and snow cover) can be described as fast feedbacks, because they respond rapidly to climate change and are thus important on decadal timescales.

Recently, ice cores (see Box 2) from the Greenland^{20,21} and Antarctic^{22,23} ice sheets and, in particular, the Vostok record²⁴⁻³⁰, which spans a full glacial-interglacial cycle²⁶, have allowed documentation of the relationship between greenhouse radiative forcing (CO₂ and CH₄) and climate over a period of large climate changes. These new data support the orbital theory of ice ages, but they are also directly relevant to the problem of greenhouse gases and climate sensitivity. Here we compare information about climate sensitivity derived from ice cores with the GCM results. Although a full understanding of the causes and sequences of climate fluctuations remains elusive, much information is revealed from comparison of glacial-interglacial palaeotemperature data with concurrent changes in the planet's energy balance that are linked with atmospheric CO₂ and CH₄ concentrations.

The ice-core data suggest that greenhouse gases have had a significant role in explaining the magnitude of past global temperature changes and indicate a net feedback for fast processes of $f \approx 3$, consistent with a climate sensitivity of $\sim 3-4^\circ\text{C}$ for a doubled atmospheric CO₂. Studies of palaeoclimate changes therefore provide a clue to help validate estimations of the climate impact of increasing greenhouse gases.

Ice-core data

Ice-core data are unique in providing, in the same samples,

Box 2 Ice cores, climate and atmospheric data

Although we cannot expect to find an ideal record of climate, ice is a rather close approximation to it as summarized in the table below⁶⁸.

CLIMATE FACTORS AND QUANTITIES MEASURED IN ICE CORES

Atmosphere	Ice core
Temperature	D/H, ¹⁸ O/ ¹⁶ O
Precipitation	D/H, ¹⁸ O/ ¹⁶ O, ¹⁰ Be
Humidity	D/H, ¹⁸ O/ ¹⁶ O
Aerosols	
natural (continents, sea, volcanoes, biosphere)	chemicals (Al, Ca, Na, H, SO ₄ , NO ₃)
man-made	SO ₄ , NO ₃ , Pb, radioactive
Circulation	fallout particles
Gases: natural and man-made	O ₂ , N ₂ , CO ₂ , CH ₄ , N ₂ O

Palaeotemperature reconstruction is based on the present correlations between the ratios of ²H (D) and ¹H and of ¹⁸O and ¹⁶O in the snow and the temperature conditions just above the inversion layer, where the precipitation is formed, and at the surface. These are correlated because of the fractionation processes that take place during the atmospheric water cycle and, although they depend on several parameters, lead to linear isotope-temperature relationships in both polar ice sheets^{69,70}. The validity of using the present observed relationship for palaeotemperature reconstruction is supported by various evidence including atmospheric isotope models⁷¹. Although there is no doubt that the concentration of a species in the air is reflected in snow deposits at the site, quantitative estimation of atmospheric concentrations suffers from an incomplete understanding of the deposition processes, such as the relative contribution of 'wet' and 'dry' processes. In contrast, relating gas concentrations obtained from ice-core bubbles to the atmospheric value is quite straightforward. Owing to the air-enclosure process in the ice, however, the air is isolated from the atmosphere well after the precipitation has been deposited; in a site of low accumulation like Vostok the correction ranges from 3 to 6 kyr with an uncertainty of up to ~ 1 kyr^{24,25}.

Adequate site selection is required to avoid disturbance of the atmospheric record by ice flow. Current deep-drilling projects to obtain long-term records include US and European efforts in central Greenland; hopefully this will supplement the few ice cores available now and provide the awaited counterpart of the Vostok Antarctic record needed for a better understanding of glacial-interglacial forcings and feedbacks.

access to both climate and climate forcings (Box 2). The climate information includes an estimate of temperature changes at the atmospheric level where the snow formed and at the surface, and the information about climate forcing includes data on the amount of aerosols in the atmosphere and the atmospheric chemical composition. So far, there are only two deep ice cores from Greenland (Camp Century²⁰ and Dye 3²¹) and three from Antarctica (Byrd²², Dome C²³ and Vostok²⁴⁻³⁰) which provide long-term climate and environmental records extending over the last ice age. Only Vostok provides a complete undisturbed series over the last climate cycle. The principle findings obtained from these deep ice cores have been recently reviewed³¹.

The Vostok temperature record is reconstructed from the continuous deuterium profile measured along the core²⁷. Here we refer to the atmospheric temperature change, ΔT_a , just above the inversion layer (Box 2) because this is the parameter that is most directly accessible from the snow deuterium content, which depends on the temperature of formation of the precipitation (such an interpretation is well supported by a theoretical approach) and because this temperature is certainly more relevant for characterizing the global atmosphere as the existence of a strong inversion is restricted to central Antarctica. The record (Fig. 1b) shows the last two glacial-interglacial transitions with atmospheric temperature changes of $\sim 6^\circ\text{C}$. The last ice age is characterized by three minima, around 20, 60 and 110 kyr BP, separated by slightly warmer episodes (interstadials).

The three Antarctic ice cores yield remarkably similar isotope-derived temperature records over the last ~ 60 kyr (ref. 32). The surface warmings associated with the last deglaciation are quite comparable with values slightly below 10°C and the Vostok record can be considered to be representative of a large area in Antarctica. The deglaciation warming for Dye 3 is similar to the one observed in Antarctica (whereas the Camp Century core suggests a somewhat larger change) and in fact, the main features of the Vostok climate record are of global significance, at least qualitatively speaking. This significance is suggested by the comparison with the global ice volume changes derived from isotope measurements in deep-sea sediments (during glacial periods the accumulation of isotopically depleted ice over the continents leads to a measurable isotope enrichment of the ocean). In particular the SPECMAP record of marine ¹⁸O content³³, which essentially represents global ice volume (Fig. 1c), and the Vostok temperature record corresponds very closely (correlation coefficient, $r^2 = 0.87$) down to 110 kyr BP²⁷. Further back there is a discrepancy in the start and in the duration of the previous interglacial which can probably be explained by dating uncertainties for this part of the record (see discussion below).

The close association between greenhouse gases and glacial-interglacial climate changes was first revealed from the analysis of air trapped in Greenland and Antarctic cores showing that the atmospheric CO₂ content was $\sim 190-200$ p.p.m.v. during the Last Glacial Maximum compared with an average close to $270-280$ p.p.m.v. during the Holocene³⁴⁻³⁷. The detailed reconstruction from Byrd³⁸ for the period between 50 and 15 kyr fully confirmed the association between cold climate and low atmospheric CO₂ levels (Fig. 2a). The Vostok data confirmed the existence of a strong CO₂-climate relationship over a full climate cycle²⁴. This CO₂ record exhibits large changes between two levels that are centred near 190-200 and 260-280 p.p.m.v., with the low and high levels associated with full glacial and interglacial conditions, respectively (Fig. 1a). Moreover, despite some noticeable differences, there is a remarkable correlation ($r^2 = 0.79$) between atmospheric CO₂ and temperature change throughout the record.

Such a correlation between climate change and radiatively active gases also exists for methane. Analysis of Dye 3 and Byrd samples showed an increase in the CH₄ concentration from 0.35 p.p.m.v. for the Last Glacial Maximum to Holocene values of 0.65 p.p.m.v.³⁹, and a similar increase paralleling the tem-

perature change was revealed from the Vostok ice for the transition between the penultimate ice age and the last interglacial²⁹. These results are now fully confirmed³⁰ by the detailed and continuous CH₄ profile obtained along the Vostok core (Fig. 1c), which also exhibits four well marked maxima during the glacial period. These maxima occur at the time of relatively warm interstadials and although there are marked differences between the CH₄ and CO₂ record, the correlation with the temperature record is quite similar ($r^2=0.78$ in the case of CH₄).

Superimposed on these long-term trends, rapid and large changes have been documented both for CO₂ and CH₄. In the time interval between 40 and 30 kyr a series of rapid CO₂ variations (Fig. 2b), occurring in a century or less between low values in the range 180–200 p.p.m.v. and higher values in the range 240–260 p.p.m.v., were found first in Dye³⁰. The existence of these rapid events, associated with large changes in surface temperature (5–6 °C) and in the dust content, has been confirmed in the Camp Century core but has not been identified in the Antarctic Byrd core, a difference not yet satisfactorily explained. There is also a clear indication of rapid climate change at the end of the last deglaciation extensively documented from isotope and dust studies on Greenland cores⁴¹. This so-called Younger Dryas cooling dated at ~11–10 kyr BP is well marked in North Atlantic and adjacent regions. Although there is also a weak cooling in Antarctica the dating is not yet sufficiently accurate to allow it to be firmly related to the Younger Dryas. Recent Vostok measurements³⁰ show that the Antarctic cooling was associated with a large decrease in CH₄ concentration (Fig. 1c), as is also suggested in the data from the Dye 3 core³⁹.

Reliable N₂O data can also be obtained from ice cores. Existing results suggest that N₂O concentrations during the Last

Glacial Maximum were possibly smaller but not drastically different from those of the recent period⁴². We can therefore estimate the direct radiative forcing exerted by all naturally occurring greenhouse gases (except changes in water vapour and possibly in ozone). In terms of radiative forcing such changes in the concentration of greenhouse gases are important because, for example, of the logarithmic increase with CO₂ and CH₄ concentrations⁴³. From glacial to interglacial this forcing increases by ~2 W m⁻² (ΔT_e of ~0.7 °C), half of that corresponding to a CO₂ doubling from 300 to 600 p.p.m.v.

Ice-core data and the theory of ice ages

The long Vostok ice core offered a unique opportunity to examine a continental record of the link between a time series for atmospheric temperature and the astronomical theory of the ice ages. Among the orbital parameters the obliquity of the Earth's axis (period of ~41 kyr) and the precession of the equinoxes (periods of 23 and 19 kyr) are the most important as they strongly affect the distribution of available energy between latitudes and seasons⁴⁴; for example, the variation of the summer insolation at 65 °N, a key latitude in the astronomical theory of the ice ages, is ~60 W m⁻² over the last climate cycle. Spectral analysis shows that the Vostok temperature record is indeed dominated by a strong obliquity component and influenced to a lesser degree by the precession²⁷. Aside from the interval of ~100 kyr between interglacials, the main features of the Vostok temperature record show that the minima are in good agreement with those of the annual insolation received at the Vostok latitude (78° S) and that there are also similarities with the 65° N summer insolation. These results further support the role of orbital forcing in Pleistocene glacial-interglacial cycles demonstrated by deep-sea sediment records⁴⁵. The orbital forcing is, however, relatively weak when considered on an annual globally averaged basis (the total insolation received by Earth has varied by <0.7 W m⁻² over the past 160 kyr). The amplification of this forcing, the observed dominant 100-kyr cycle and the synchronized termination of the main glaciations and their similar amplitude in the Northern and Southern Hemispheres cannot easily be explained despite developments including the nonlinear response of ice sheets to orbital forcing⁴⁶. The discovery of significant changes in climate forcing linked with the composition of the atmosphere has led to the idea that changes in the CO₂^{24,28,47–49} and CH₄^{29,30} content have played a significant part in the glacial-interglacial climate changes by amplifying, together with the growth and decay of the Northern Hemisphere ice sheets, the relatively weak orbital forcing and by constituting a link between the Northern and Southern Hemisphere climates.

The observed changes in CO₂ and CH₄ imply modifications in their sources and sinks that probably involve very different processes such as ocean circulation and marine production for CO₂ (see ref. 50 for a review) and fluxes of emission from natural wetlands for CH₄^{29,30}. The mechanisms behind these modifications are not yet fully understood, especially those involving CO₂, but we note that the orbital frequencies are present in both of these Vostok series with, in particular, a strong precessional signal^{24,30}. This suggests that the changes are in some way orbitally driven and, even if the orbital forcing is not the only important effect, this supports the idea that orbital changes are one initial cause of the ice ages.

Further, the astronomical theory cannot easily explain the rapid events recorded in ice cores. Rather, although the mechanisms are still unknown, rapid changes could be connected to a flip-flop mechanism in the North Atlantic ocean, perhaps a turning on and off of the North Atlantic current⁵¹. We note, however, that the correspondence between cold episodes and low CO₂ concentration (Fig. 2b) and conversely between warmest periods and high concentrations seems to hold true during the last ice age and possibly during the Younger Dryas

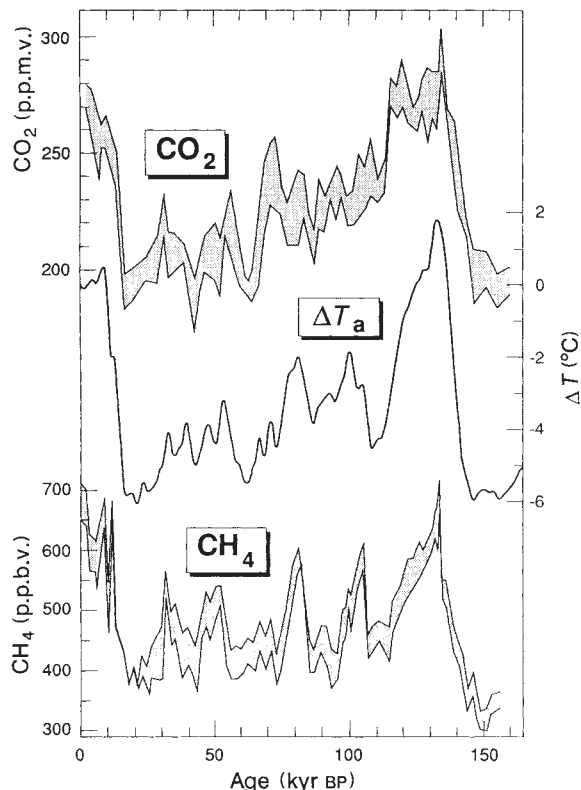


FIG. 1 Variation during the last climate cycle, as derived from measurements along the 2,083-m Vostok ice core, of the CO₂ atmospheric concentration²⁴, the atmospheric temperature change over Antarctica²⁷, and the CH₄ atmospheric concentration³⁰. For CO₂ and CH₄ the envelope shown has been plotted taking into account the different sources of uncertainty whereas the temperature record is a smoothed curve.

event, as suggested by the CH₄ data (Fig. 1c). There is therefore some indication that greenhouse forcing also participated in the amplitude of these temperature changes.

Ice-core data and the greenhouse effect

The glacial-interglacial changes include not only the fast feedback processes associated with water vapour, cloud properties, snow cover and sea ice but also longer-term processes such as those linked with slow changes in several boundary conditions and in the atmospheric composition, which help maintain the ice age cold relative to the interglacial (Box 1). We now derive information about the role of these slow changes from the Vostok temperature series and compare it with other palaeodata and climate forcings. Using the data on the direct radiative forcing associated with changes in the concentration of greenhouse gases, we derive information about the role of fast feedback processes. This does not require a solution of the 'chicken and egg' problem, that is, we do not have to address fully the question of causes of the glacial-interglacial cycles and of the sequence of possible forcing factors^{28,52}. For example, whether the temperature changes lead or lag the changes in CO₂ or CH₄ concentrations is not relevant for the study of fast feedbacks. There is, however, the important constraint that the planet must be in near radiation balance with space. This may require several thousand years and we therefore cannot use this approach to address the question of transients or rapid events, which may occur in a few decades⁴¹. Within these limits, we may assume that greenhouse gases have contributed to the glacial-interglacial temperature change through their direct radiative forcing associated with fast feedback processes. As discussed below, such an assumption is well supported by the GCM experiments described in refs 49 and 53 and we can take advantage of the long records of climate forcings and climate outputs, which reflect equilibrium conditions, to evaluate (or at least reasonably bracket) the role of greenhouse-gas radiative forcing in determining past climate.

Genthon *et al.*²⁸ followed such an approach in performing a multivariate analysis of the Vostok temperature record and three climate inputs. This approach decomposes the temperature variance into a part that can be accounted for by the inputs plus a residual, which is due to noise not related to these inputs. The relative contribution of each input is calculated so that the noise

is minimized in a least-squares sense⁵⁴. To be able to partition properly the output series as a combination of the input series, the input series must be independent. For climate forcing, Genthon *et al.*²⁸ used CO₂, a Southern Hemisphere component represented by the local (78° S) annual insolation (Fig. 3d) and a Northern Hemisphere component derived either from the 65° N July insolation or from the $\delta^{18}\text{O}$ marine record, a proxy of the Northern Hemisphere ice volume (Fig. 3c). We have used this approach and extended it to account for the following points.

First, such a multivariate analysis implicitly assumes that the system can be described by its steady-state behaviour. This is not the case for a climate system affected by nonlinearities associated with the growth and decay of the ice sheets. To account for this, we consider cases in which the ice volume reconstructed from the $\delta^{18}\text{O}$ record is taken as a proxy of the Northern Hemisphere forcing. This proxy combines the orbital insolation forcing and the nonlinearities mentioned above. We can therefore assume that it represents a large part of the slow feedbacks associated with the changes in boundary conditions, all the more because the increased area of ice sheets provides a large forcing ($\sim 3 \text{ W m}^{-2}$ mainly owing to albedo effects but also to topographic changes).

Second, we account for changes in both CO₂ and CH₄ to evaluate the combined greenhouse radiative forcing. For this purpose we use the CO₂ and CH₄ Vostok records (including data from other cores would not result in significant changes, except for rapid events not considered here) and calculate the associated direct radiative forcing through the formulae given by Hansen *et al.*⁴³. For CH₄, this direct radiative forcing was augmented by a factor of ~ 2 because of chemical feedbacks linked with the increase of stratospheric water vapour³⁰. The total greenhouse radiative forcing expressed in terms of equilibrium temperature change (with no climate feedback) given in Fig. 3b shows a glacial-interglacial amplitude of $\sim 0.7^\circ\text{C}$.

Third, we found it useful to explore a wider range of possible atmospheric radiative forcings such as those resulting from changes in the aerosol loading^{18,55} and in the amount of cloud condensation nuclei (CCN). A strong increase (up to a factor of 30) in the dust fallout has been recorded for the Last Glacial Maximum both in Greenland and Antarctic cores, as illustrated in Fig. 2c for Dome C⁵⁶. It has been claimed that the resulting increase in the optical depth of the atmospheric aerosols could make a significant contribution to the cooling during this period¹⁸. This is still uncertain because it is difficult to evaluate the global aerosol loading using data coming mostly from polar ice cores. Also, a change in the optical depth and thus in direct radiative forcing depends strongly on the dust optical properties, which are not known. Analysis of the Vostok core⁵⁷ showed a strong dust increase at the end of the penultimate ice age and during the cold interstadial centred around 60 kyr BP, which also indicates a relation between cold periods and high aerosol loading. The Vostok dust record is very spiky, however, and not simply related with the smoother temperature record. There is no dust peak during the cold period around 110 kyr and the correlation ($r^2 = 0.36$) with the temperature record is much lower than the corresponding one for greenhouse gases. It has recently been proposed that the number concentration of CCN, mainly sulphate particles produced by the oxidation of dimethylsulphide emitted from the ocean, influences the albedo of marine stratus cloud and hence global climate⁵⁵. In this hypothesis, a change in marine productivity may eventually act as a climate forcing through the induced change in CCN concentration. Using the Vostok non-sea-salt sulphate as a proxy for CCN concentration, Legrand *et al.*⁵⁸ suggested that this forcing may have participated in the glacial cooling. The correlation between non-sea-salt sulphate and the Vostok temperature is relatively high ($r^2 = 0.63$) but it must be noted that the role of CCN and the CCN-sulphate link are not yet firmly established.

TABLE 1 Results from recent doubled-CO₂ models

Study	Source	ΔT_s	f
Goddard Institute for Space Studies National Center for Atmospheric Research	ref. 6	4.2	3.5
Geophysical Fluid Dynamics Laboratory	ref. 7	4.0	3.3
UK Meteorological Office	ref. 8	4.0	3.3
Oregon State University	ref. 9	5.2	4.3
UK Meteorological Office	ref. 10	2.8	2.3
	ref. 11	1.9	1.6

Most results are adapted from ref. 1; an extreme experiment⁴¹ in which cloud cover and cloud radiative properties depend strongly on temperature cloud water content is also included. The values of ΔT_s obtained by the GCMs, 1.9–5.2 °C, are substantially larger than the values of ΔT_e , indicating that the modelled processes significantly amplify the direct greenhouse forcing. More general discussions of the equilibrium climate sensitivity relevant to short timescales fall in the range $\Delta T_s = 1.5\text{--}5.5^\circ\text{C}^{1-3,14,15}$. Thus the uncertainty in f seems to be $f \approx 1\text{--}4$. There are other potentially important feedbacks, which are not investigated by the GCM studies because they are treated as either fixed boundary conditions or as specified climate forcings. Many of these could be classified as slow feedbacks which may be significant on long timescales, such as glacial-interglacial climate change. Examples include changes of land ice, floating ice shelves⁶⁶ vegetation cover⁶, and ocean circulation⁶⁷. Possible atmospheric changes include the abundance of greenhouse gases, dust¹⁸ and cloud condensation nuclei⁵⁵, including the impact of these on optical properties of clouds¹⁸.

Finally, we reexamined the previously noted influence of the dating²⁸. Indeed, the multivariate analysis must be done with all time series having a common timescale. This is not a problem for the temperature, non-sea-salt sulphate and dust Vostok series and, within the uncertainty associated with the correction due to the air-ice difference (Box 2), for the greenhouse forcing. There is, however, a noticeable dating discrepancy between the Vostok and ice volume records before 110 kyr, which has been shown to affect significantly the results of the multivariate analysis performed by Genthon *et al.*²⁸. We tested numerous redatings of the oldest part of the Vostok series accounting for the recent interpretation of the Vostok dust record, which suggests that the marine and Vostok records are roughly in phase before 110 kyr⁵⁷. A new multivariate analysis was then performed using five forcing factors: greenhouse gases, dust and non-sea-salt sulphate concentrations, ice volume and local insolation. The results may be summarized as follows:

There is no case for which the contribution of the greenhouse effect is below 40%. The lowest calculated value is 42% when the Vostok record is put exactly in phase with the marine $\delta^{18}\text{O}$ record, with a similar contribution from the ice volume (45%) and with contributions of $\leq 5\%$ for each of the three other climate inputs. With this redating, Genthon *et al.*²⁸ obtained a CO_2 contribution of 35%. Indeed, for all analyses performed, adding CH_4 leads to a $\sim 10\%$ increase over the effect derived using only CO_2 . This may be related⁵⁹ to the higher correlation of the Vostok temperature with the combined greenhouse ($r^2 = 0.84$) than with either CO_2 ($r^2 = 0.79$) or CH_4 ($r^2 = 0.78$).

Within the limits of dating by comparison of the Vostok dust record with marine records, the greenhouse contribution never exceeds 65%, with the sum of greenhouse and Northern Hemisphere forcing being generally over 80%. Generally $>90\%$ of the variance of the Vostok temperature is explained by the five climate inputs.

The greenhouse contribution falls in the top of this 40–65% range (between 55 and 65%) when the ice volume record of Labeyrie *et al.*⁶⁰ is used, instead of the SPECMAP curve, to represent the Northern Hemisphere forcing. This alternative approach was followed because the Labeyrie *et al.* curve probably represents the ice volume change better than the more generally used SPECMAP record, because it is corrected for the part of the isotope signal that comes from the change in ocean temperature. Also, there may be, at least for CO_2 ²⁴, some link between sea level (or ice volume) and the change in the atmospheric concentration and using different ice-volume curves

is one way to estimate the bias introduced in the multivariate analysis by two of the input series (greenhouse and ice volume) not being fully independent.

To summarize, the contribution of greenhouse gases to the Vostok temperature change can be bracketed between a lower estimate of 40% and a higher estimate of 65%. This range is somewhat uncertain, however, owing to the nonlinearities linked with the growth and decay of the Northern Hemisphere ice sheet, all the series not having a common timescale and the possibility that there may have been other important forcings that we have not taken into account. We have attempted to account for the first two factors. In addition, the introduction of a new climate input, which must correspond to a process having a potential climate input, will significantly alter the result of the multivariate analysis only if this new series is well correlated with the Vostok temperature and independent of the other inputs. One example of such a forcing is that linked with vegetation change through its influence on the albedo, but model results^{43,49}, show that the climate role of the vegetation is relatively minor in maintaining the glacial climate. Although we cannot rule out another forcing, it is difficult to imagine another meeting these three requirements. So, within the limits of our multivariate analysis, $\sim 50 \pm 10\%$ is a reasonable estimate for the overall contribution of the greenhouse gases to the Vostok temperature change over the last climate cycle. This means that $\sim 3^\circ\text{C}$ of the 6°C in the glacial-interglacial change at Vostok could be attributed to the greenhouse effect. This result agrees well with the GCM simulations for the Last Glacial Maximum performed by Broccoli and Manabe⁴⁹. Using a model with prescribed cloud cover, they reduced the CO_2 concentration from 300 to 200 p.p.m.v. in experiments with and without changes in other boundary conditions. The CO_2 change alone, which in terms of radiative forcing roughly corresponds to the combined CO_2 and CH_4 glacial-interglacial change measured in ice cores, results in a temperature drop of $2\text{--}2.5^\circ\text{C}$ in central east Antarctica. With variable cloud, however, the GCM sensitivity is increased by 31% ¹⁹ implying a change of $\sim 3^\circ\text{C}$ over this part of Antarctica. Although based on one particular GCM and experiment, this agreement between two independent approaches is worth noting.

Ice-core data and climate sensitivity

Despite the large geographical significance of the Vostok temperature record, it can be argued that looking at one particular site or area is too restrictive. Other palaeoclimate data and climate simulations are indeed helpful in examining the problem

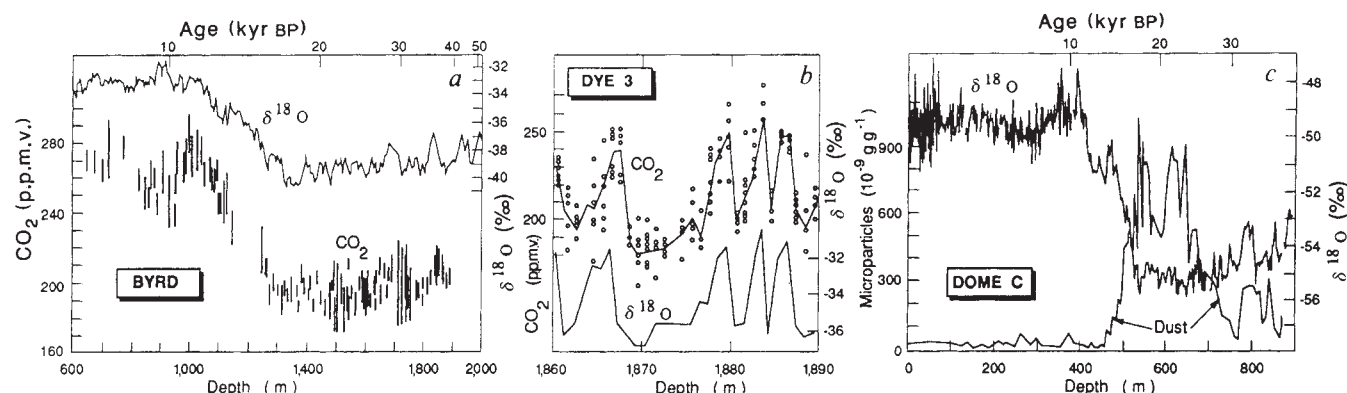


FIG. 2 This panel shows key data obtained from the Byrd (West Antarctica), Dye 3 (Greenland) and Dome C (East Antarctica) cores. The results are given as a function of depth. The corresponding timescale (upper abscissa) is also given, except for Dye 3, for which the 30-m ice increment corresponds to approximately 10 kyr between 40 and 30 kyr. The $\delta^{18}\text{O}$ record has been reported for each of these three cores ($\delta^{18}\text{O}(\text{‰}) = \{[(^{18}\text{O}/^{16}\text{O})_{\text{sample}} / (^{18}\text{O}/^{16}\text{O})_{\text{standard}}] - 1\} \times 1,000$ where the standard is

standard mean ocean water. a, Byrd: A detailed CO_2 record covering the last glacial period and part of the Holocene³⁸. b, Dye 3: Rapid CO_2 changes (circles indicate the results of single measurements and the upper solid line connects the mean value for each depth), adapted from ref. 40. c, Dome C: The Dome C dust record showing a strong increase of dust during the Last Glacial Maximum⁵⁶.

of greenhouse gases and climate sensitivity in a global perspective.

First, we note that owing to the general polar amplification of climate changes, the Vostok inferred glacial-interglacial warming of $\sim 6^\circ\text{C}$ over Antarctica is consistent with a globally averaged value of $\sim 4\text{--}5^\circ\text{C}$; for example, in doubled- CO_2 GCM experiments this amplification is, on average, $\sim 50\%$ for high-latitude continental regions³. This indicates that we can extend the conclusion that the greenhouse effect resulted in $\sim 50\%$ of the temperature change in the last climatic cycle to be a global estimate. This is supported by the similarities observed between the Vostok temperature and palaeorecords of global character; the multivariate analysis shows that generally $>80\%$ of the explained Vostok temperature variance is due to climate forcings of global significance (ice volume and greenhouse gases). Similarities between the Vostok and other palaeotemperature records further support this conclusion. For example, the Vostok temperature is well correlated with the surface temperature at the Indian Ocean site RC11-120³⁴ (with $r^2 = 0.66$ for the period before 110 kyr for which redating uncertainties are not critical). Replacing the Vostok temperature record by the RC 11-120 record as climate output in the multivariate analysis left a contribution of the greenhouse gases to the explained variance of $\geq 50\%$.

The approach used here suggests that greenhouse gases contributed $\sim 2^\circ\text{C}$ to the global warming between glacial and interglacial periods. Estimating climate sensitivity then simply consists in dividing the estimated global warming associated with the greenhouse forcing by the corresponding ΔT_e (0.7°C from glacial to interglacial) to get the net feedback factor. We stress

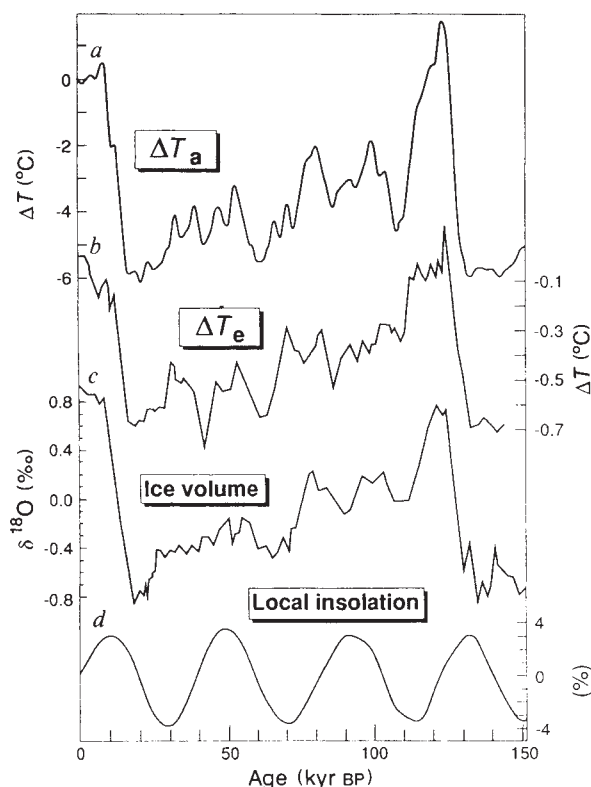


FIG. 3 Time series of the Vostok climate record and of climate forcings used in the multivariate analysis: a, atmospheric temperature change over Antarctica (Vostok record), ΔT_a ; b, the direct greenhouse radiative forcing accounting for CO_2 and CH_4 variations, ΔT_e ; c, $\delta^{18}\text{O}$ SPECMAP record taken as a proxy of the change in ice volume (normalized value from ref. 33); d, percentage change in total insolation at the Vostok latitude (78°S) during the entire year. Vostok curves (a and b) have been redated for the period before 110 kyr in such a way to put the Vostok temperature and the marine records in phase.

the independence of this method with respect to that based on GCM results. Although GCM simulations are useful in providing evidence that the regional results have a global significance, this use does not make our reasoning circular.

Second, it is of interest to compare the relative contribution of the greenhouse forcing as independently derived from the multivariate analysis and from GCMs. These results, only available for the GFDL and GISS models, indicate that a significant part of the glacial-interglacial warming may, on a global scale, be attributed to the greenhouse effect. In the Broccoli and Manabe experiments⁴⁹ the CO_2 increase from 200 to 300 p.p.m.v. and associated fast feedbacks, accounts for $\sim 40\%$ of the global glacial-interglacial warming. These experiments also showed that most of the remaining warming is due to the retreat of the Northern Hemisphere ice sheet in agreement with our multivariate analysis: Using the GISS model, Rind *et al.*⁵³ recently mentioned that an average warming of 2.5°C would result from a CO_2 increase from 230 to 300 p.p.m.v. This corresponds to at least $\sim 50\%$ of the glacial-interglacial warming predicted by the GISS model for various simulations of the Last Glacial Maximum⁶. Such agreement supports the belief that the net effect of the fast feedback processes which amplify the direct greenhouse radiative forcing, or at least their combined effect, are realistically evaluated in these two models (or in models having similar climate sensitivity).

To the extent that atmospheric feedback processes are independent of the detailed causes of climate changes but governed by the overall climate response, the same feedback processes would operate now as during the Last Glacial Maximum¹⁸. Applying the net feedback factor of three deduced from ice-core data to the present climate may be done without further assumptions (at least within the limits of uncertainty of our method for estimating f). For greenhouse model experiments, climate sensitivity depends primarily on fast feedback processes. Analysis of ice-core results and palaeodata over a full glacial-interglacial cycle suggests that a warming induced by doubled CO_2 concentrations of $3\text{--}4^\circ\text{C}$ ($f \approx 3$) may be a realistic value which, although being in the middle of the range of values inferred from the GCM experiments (Box 1), corresponds to a relatively high climate sensitivity.

Concluding remarks

Ice-core records have been important in determining that climate and greenhouse-gas concentrations were intensely interactive. Palaeoclimate reconstructions are now recognized as an important element in climate studies because they: (1) allow us to assess the degree of natural variability and put observed changes in present climate in a broader perspective; (2) assist in understanding the causes and mechanisms of climate change (by relating climate and forcing factors, for example); (3) contribute to validating models through the comparison of outputs with empirical data sets; (4) may potentially be used in predicting geographical patterns of future climate based on past analogues.

We have approached the evaluation of climate sensitivity to greenhouse gases from the point of view of both the modellers and palaeoclimatologists. Despite the relative agreement of the two approaches there is still much research to be done and our assertion about the irrelevance of fully addressing the causes of glacial-interglacial cycles for estimating climate sensitivity must not be misunderstood. We attach the utmost importance to a full understanding of the physical, chemical and biological processes by which subtle changes in insolation are amplified to induce long-term changes in global climate. For this, a knowledge of the sequence of events and the exact timing of forcings and of the climate responses in various parts of the Earth system is essential. This includes acquisition of new well dated series from land, ocean and ice records to document fully the changes that have affected our planet over the last climate cycle; obtaining a globally averaged temperature record would improve our multivariate approach.

As far as ice cores are concerned, there is still a lot of information directly relevant to the interaction of greenhouse gases and climate buried in both Antarctic and Greenland polar ice caps. Obtaining Northern Hemisphere ice records of climate, greenhouse gases and other climate forcings over a full glacial-interglacial cycle is one important objective for a better understanding of the past complex interactions between Northern and Southern Hemispheres on this timescale. This objective is part of the GRIP (Greenland Ice Core Project) and GISP II (Greenland Ice Sheet Project) now being conducted in north central Greenland by European and American scientists. These drillings are expected to reach the bedrock (the ice thickness is 3.2 km) in 1992 and to cover the last climate cycle and hopefully more. These cores will allow further documentation of the rapid climate changes discussed here. With a snow accumulation higher than at Vostok they should also allow a better determination of the relative timing (phase lag) of climate and greenhouse forcing. Plans are also being developed for further drilling in Antarctica. Drilling at Vostok was interrupted at a depth of 2,546 m but Soviet drillers are starting a new hole with the aim of reaching the bedrock, that is, obtaining a 3.5-km ice core. Several other nations are also planning deep drilling in Antarctica (Australia, France, Japan, USA, for example). Scientists and operating agencies should develop a plan to organize an array of shallow, intermediate and deep ice cores ensuring both proper geographical coverage and coverage of relevant timescales at appropriate resolution. These plans should aim to obtain records of climate and forcing factors but also address other problems such as the interaction between climate and ice sheets and the

role of the ice-sheet mass balance in sea level changes.

A strong interaction between data acquisition and interpretation and modelling of palaeoclimate and palaeoenvironment changes including the atmosphere, ocean, cryosphere, hydrosphere and land processes and various biogeochemical cycles is also needed. As well as GCMs, which produce steady-state simulations, a hierarchy of time-dependent models ranging from simple or even conceptual^{46-48,61,62} to more complex two-dimensional models⁶³ can certainly provide information on how the various processes combine to produce observed climate changes. For this, understanding mechanisms that cause and are associated with rapid climate change is crucial. The effort in these directions must be well supported and the fact that the study of glacial-interglacial cycles constitutes one of the streams of the IGBP (International Geosphere Biosphere Program) project on Global Changes of the Past (PAGES) is very positive. Further studies of climate change will narrow the uncertainties in our present approach of estimating climate sensitivity. A modelling effort coupled with interpretation of palaeodata provides, along with the satellite data on radiative forcing⁶⁴, perhaps our best hope in the near future for predicting the impact of increasing greenhouse gases on climate. □

C. Lorius and D. Raynaud are at the Laboratoire de Glaciologie et Geophysique de l'Environnement, BP 96, 38402 St Martin d'Heres Cedex, France. J. Jouzel is at the Laboratoire de Geochimie Isotopique, CEA/DSM Centre d'Etudes Nucleaires de Saclay, 91191 Gif sur Yvette Cedex, France. J. Hansen is at the NASA/Goddard Space Flight Center's Institute for Space Studies, 2880 Broadway, New York, New York 10025, USA. H. Le Treut is at the Laboratoire de Meteorologie Dynamique, 24 Rue Lhomond, 75234 Paris Cedex, France.

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Molecular cloning and characterization of a novel dopamine receptor (D₃) as a target for neuroleptics

Pierre Sokoloff*, Bruno Giros*, Marie-Pascale Martres*, Marie-Louise Bouthenet† & Jean-Charles Schwartz*

* Unité de Neurobiologie et Pharmacologie (U. 109) de l'INSERM, Centre Paul Broca, 2ter rue d'Alésia, 75014 Paris, France

† Laboratoire de Physiologie, Faculté de Pharmacie, Université René Descartes, Paris, France

A dopamine receptor has been characterized which differs in its pharmacology and signalling system from the D₁ or D₂ receptor and represents both an autoreceptor and a postsynaptic receptor. The D₃ receptor is localized to limbic areas of the brain, which are associated with cognitive, emotional and endocrine functions. It seems to mediate some of the effects of antipsychotic drugs and drugs used against Parkinson's disease, that were previously thought to interact only with D₂ receptors.

DOPAMINE affects its target cells in brain and endocrine tissues by interacting with D₁ and D₂ receptors^{1,2}. These two receptor subtypes differ in their pharmacological specificity with neuroleptics, a class of dopamine antagonists^{3,4} used to alleviate the main symptoms of schizophrenia⁵ which all display nanomolar potency at the D₂ receptor^{6,7} but variable potency at the D₁ receptor⁸. Also, stimulation of D₁ receptors increases adenylyl cyclase activity but D₂ receptor stimulation reduces it⁹, and the two receptor subtypes are differently distributed among cerebral neurons¹⁰⁻¹² and endocrine cells².

This categorization might be incomplete as, for instance, the binding of D₂ receptor ligands to cerebral membranes seems heterogeneous^{13,14}, although this remains controversial¹⁵. Also, autoreceptors¹⁶ expressed by dopamine neurons, which mediate inhibition of neuron activity, as well as of dopamine synthesis or release may differ pharmacologically from postsynaptic D₂ receptors^{17,18}, however, following the cloning of a D₂ receptor complementary DNA¹⁹, two isoforms of the receptor, termed D_{2A} and D_{2B} (or D₂₍₄₄₄₎ and D₂₍₄₁₅₎) that display the same pharmacology, were shown to be synthesized by both dopamine and postsynaptic neurons²⁰⁻²³. Finally, the ability of neuroleptics to block various behavioural characteristics elicited by dopamine agonists in rodents^{14,24-26} and to alleviate psychotic symptoms differs from their ability to cause parkinsonian side-effects in psychiatric patients²⁷, but this might reflect differential distributions among brain regions²⁷ or interaction with various receptors other than dopamine²⁸. Hence, the identification of additional dopamine receptor subtypes, distinct from the D₁ and D₂ receptors has remained elusive, in spite of its potential value, particularly in psychiatry, where the design of functionally more discriminatory drugs than the available neuroleptics is highly desirable.

We describe here the molecular cloning and expression of a full-length cDNA for a novel dopamine receptor belonging to the superfamily of G protein-coupled receptors that is encoded by a gene homologous to the D₂ receptor gene. It encodes a receptor with a pharmacology, tissue distribution and, presumably, a transduction system that differ from those of either D₁

or D₂ receptors. Therefore, we have termed it the D₃ receptor and discuss how its characteristics might account for the antipsychotic and other known properties of neuroleptics.

Molecular cloning and structure

The cDNA was cloned by screening of cDNA and genomic libraries and a combination of reverse transcription and polymerase chain reaction (RT-PCR)²⁹.

A clone isolated from a rat brain cDNA library²⁰ using a probe derived from the D₂ receptor sequence¹⁹ was initially used to screen a genomic library. Sequence analysis of a positive clone (λRG 12A) indicated an open reading frame encoding a protein with strong homology with transmembrane segments (Mbs) I and II of the D₂ receptor, suggesting that it corresponded to a segment of a gene homologous to the D₂ receptor gene. A degenerated oligonucleotide from Mb VII of the D₂ receptor was used in RT-PCR with another primer located upstream from the putative ATG initiating codon of the clone λRGE 12A (primer 1 in Fig. 1a). The PCR products were subcloned, sequenced and a restriction fragment used to screen a genomic library and obtain the end of the coding region. A positive clone (λRGS 3) was partially sequenced and a specific primer located downstream of the TGA stop codon (primer 3 in Fig. 1a) was used with primer 1 in RT-PCR, the products were subcloned and sequenced (RT-PCR B in Fig. 1a).

The amino-acid sequence of the D₃ receptor was deduced from the open reading frame of this clone (Fig. 1b). The translational initiation site was assigned to the first ATG codon downstream from an in-frame nonsense codon (−69), found in λRGE 12A (GGGCCATGGCA)³⁰. As a translational termination codon (TGA) occurs in frame following codon 446, we conclude that the D₃ receptor consists of 446 amino-acid residues. It contains seven putative Mbs characteristic of all members of the superfamily of G protein-coupled receptors³¹. Like the D₂ receptor gene^{19,20,22,32,33}, but unlike most other members of this superfamily, the D₃ receptor gene contains introns. The positions of five introns are indicated in Fig. 1b, two of which correspond to introns in the D₂ receptor gene. Comparison of the sequences of clones λRGS 3 and RT-PCR B identified a single intron within the third putative intracytoplasmic loop, suggesting that generation of isoforms by alternative splicing, which takes place for the D₂ receptor²⁰⁻²³, does not occur for the D₃ receptor at this level.

The homology between the rat D_{2A} and D₃ receptors is 52% overall but as high as 75% if only the transmembrane domains—believed to be responsible for ligand recognition—are considered. Functionally significant amino-acid residues can be noted in the D₃ receptor: Asp 110, responsible for salt-linking ammonium groups of monoamines; Ser 193 and Ser 196 for hydrogen bonding of the two hydroxyl groups of catechols³⁴ and Cys 103 and Cys 181 that may participate in the formation of an extracellular disulphide bridge³⁵.

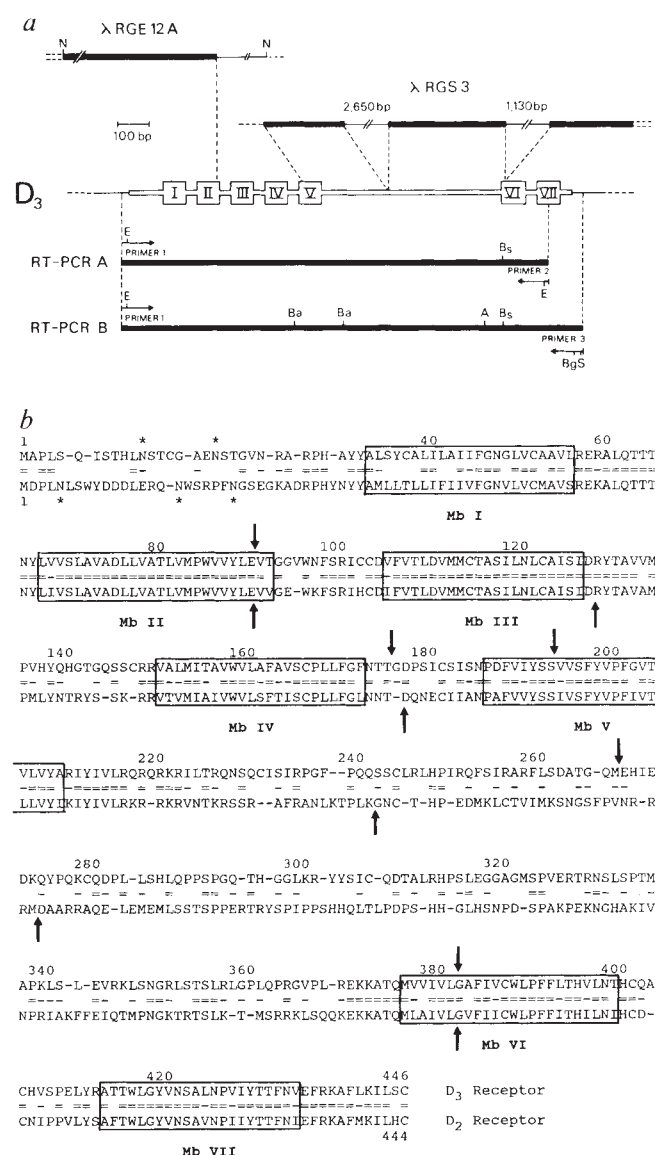
Pharmacology and signalling system

When expressed in COS-7 cells (not shown) or CHO cells, D_2 and D_3 receptors were both labelled by [125 I]iodosulpride¹¹ with dissociation constants (K_d) of 0.6 nM and 1.2 nM, respectively. The two receptors were clearly distinguished by several dopamine agonists or antagonists. Dopamine itself, in the absence of added guanylnucleotide, was about 20 times more potent at the D_3 than at the D_2 receptor (Fig. 2a and Table 1). In the presence of Gpp(NH)p, a guanylnucleotide, the dopamine displacement curve at the D_2 receptor was shifted rightward (Fig. 2a), indicating the presence of a G_i protein in the recipient CHO cell. By contrast, the dopamine displacement curve at D_3 receptors was not significantly modified by Gpp(NH)p (Fig. 2a). This may reflect the absence of a suitable G protein in the recipient CHO cell (or in the COS-7 cell, in which a similar observation was made; data not shown) or a low modulation of dopamine binding by guanylnucleotides at the D_3 receptor. A high affinity component of dopamine bind-

ing¹³, also not modulated by guanylnucleotides^{13,36}, was previously detected in crude cerebral membranes. Among dopamine agonists, apomorphine and bromocriptine displayed similar potencies at both receptors, whereas TL 99, pergolide and quinpirole—considered to be putative autoreceptor-selective agents^{9,17}—were much more potent at D_3 than at D_2 receptors. Most neuroleptics displayed nanomolar potencies at both receptors, which varied depending on the receptor. For instance, haloperidol, spiperone, thiopropazine and prochlorperazine were 10 to 20 times more potent at D_2 than at D_3 receptors, whereas (–)sulpiride, clozapine, thioridazine, amisulpride or raclopride were only 2 to 3 times more potent at D_2 than at D_3 receptors. Interestingly, the first series of compounds are considered to be 'typical' neuroleptics, that is, drugs displaying both high antipsychotic activity and high ability to cause parkinsonian side effects, whereas most compounds of the second series are considered to be 'atypical' neuroleptics, in the sense that their antipsychotic activity is less associated with

FIG. 1 Cloning strategy and amino-acid sequence of the D_3 dopamine receptor. a, Schematic representation of the organization of the clones obtained after genomic DNA screening and PCR amplification. The D_3 sequence is drawn as seven boxes representing Mbs I–VII, open boxes representing coding sequences and single lines untranslated sequences. λ RGE 12A and λ RGS 3 are genomic DNA clones, RT-PCR A and B represent cDNA clones obtained from poly(A)⁺ mRNA RT-PCR amplifications. Solid boxes, coding regions (exons of genomic DNA clones); thin lines, introns in the genomic DNA clones. Dotted lines indicate continuity of the sequence. Primers 1, 2 and 3 were used for RT-PCR. Restriction sites are: A, *Aval*; Ba, *Bam*HI; Bg, *Bgl*II; Bs, *Bst*XI; E, *Eco*RI; N, *Nhe*I and S, *Sal*I. Note that *Eco*RI, *Bgl*II and *Sal*I are adaptors at the 5' end of RT-PCR primers. b, Comparison of the amino-acid sequences (single-letter amino-acid code) of D_3 and D_2 receptors, with alignment according to ref. 43. Transmembrane regions are boxed and intron positions for rat D_2 receptor^{20,22,32} and D_3 receptor, deduced from analysis of λ RGE 12A and λ RGS 3, are indicated by arrows. = indicates amino-acid conservation and –, conservative substitutions where groups are (H, K, R), (I, L, V, M), (A, G, S, T), (Y, F, W), (D, E, P, N, B, Z), (P) and (C).

METHODS. Overlapping oligonucleotides derived from the D_{2B} dopaminergic receptor (nucleotides 146–233 in ref. 19) were radiolabelled and used to screen a rat brain cDNA library²⁰. One clone was shown to hybridize weakly with a *Bam*HI–*Bgl*II restriction fragment of D_{2A} receptor clone, but not with an *Acc*I–*Acc*I probe (nucleotides 278–571) encoding Mbs II–V. An *Aat*II–*Msc*I fragment of this clone, ³²P labelled by nick-translation, was used to screen a partial *Eco*RI-cut rat genomic library constructed in Charon 4A (from Dr A. M. Duchemin). Nitrocellulose filters with transferred phages were hybridized 16 h at 42 °C in 60% formamide, 10% dextran sulphate, 4 × SSC buffer, 8 mM Tris–HCl, pH 7.4, 1 × Denhardt's solution, 20 μ g ml^{−1} yeast transfer RNA, 20 μ g ml^{−1} salmon sperm DNA, 0.1% SDS, and probed at 7.5 × 10⁵ d.p.m. ml^{−1}. Filters were washed twice for 20 min at 42 °C in 2 × SSC, 0.1% SDS and twice for 30 min in 0.2 × SSC, 0.1% SDS at 42 °C and 55 °C, respectively. Three identical positive clones were obtained (including λ RGE 12A) over 800,000 clones and a *Nhe*I–*Nhe*I fragment was subcloned into the *Xba*I site of M13 and sequenced⁴⁴. Two primers derived from this sequence were synthesized (PCR-Mate 391A DNA Synthesizer, Applied Biosystems) at position −32 (5'-ACATTTTGGAGTCGCGTTCCTCTG, primer 4) and position −7 (CGAATTCATGGACCTCTGAGCCAGATAAGCAC, primer 1). A single-strand cDNA was synthesized using AMV-reverse transcriptase (20 U, Boehringer), from rat brain poly(A)⁺ mRNA (2 μ g) and a degenerated primer from the sequence of Mb VII of the D_3 receptor (primer 2) 5'-CGAATT-CGAC(GA)GCACTGTT(GC)AC(GA)TA(GC)CC(GC)AGCCA at 375 nM. This template was amplified²⁹ using primer 4 and primer 2 (75 nM each) for 30 cycles (92 °C, 52 °C and 72 °C for 1 min each) with 2.5 U *Taq* DNA polymerase (Perkin Elmer Cetus). After resolution of PCR products by agarose gel electrophoresis, blotting and hybridization with the *Acc*I–*Acc*I D_2 receptor probe, a reactive band at 1.3 kb was excised and the DNA extracted. This DNA was amplified again by PCR with primers 1 and 2. A band was detected after ethidium bromide staining, cut out, and the DNA extracted, digested with *Eco*RI and subcloned into M13 (clone RT-PCR A) for sequencing and into pGEM-4Z (Promega). A *Bst*XI–*Eco*RI radiolabelled restriction fragment was used to screen a partial *Sau*3A-cut rat genomic library (Clontech), as described above. A positive clone, λ RGS 3, was obtained, analysed and a 3.5 kb *Nhe*I–*Nhe*I fragment hybridizing to the *Bst*XI–*Eco*RI probe was subcloned into M13 and sequenced. A primer was synthesized 45 nucleotides downstream from the first in-frame TGA stop codon (primer 3: CCACGT-



CGACAGATCTCGAAGTGGGTAAAGGAGTG). Primers 1 and 3 were then used in RT-PCR as described using poly(A)⁺ mRNA of olfactory tubercle. A band of the predicted size (1.4 kb) was extracted after agarose gel electrophoresis and digested with *Eco*RI–*Sal*I for directional subcloning in M13 mp18, M13 mp19 and pGEM 4Z. Four individual clones in M13 were entirely sequenced and found to be identical to one another, as well as to corresponding sequences in λ RGE 12A and λ RGS 3.

TABLE 1 Pharmacology of D₂ and D₃ receptors expressed in CHO cells

Agents	K_i value (nM)		Ratio K_{iD2}/K_{iD3}
	D ₂ receptor	D ₃ receptor	
Agonists			
Bromocriptine	5.3 ± 0.6	7.4 ± 1.3	0.7
Apomorphine	24 ± 2	20 ± 3	1.2
SKF 38393	9,560 ± 408	5,000 ± 500	1.9
Dopamine	474 ± 33	25 ± 3	19
Dopamine + Gpp(NH)p	1,705 ± 270	27 ± 3	63
TL 99	18 ± 1	0.91 ± 0.08	20
Pergolide	21 ± 3	0.62 ± 0.04	33
Quinpirole	576 ± 47	5.1 ± 0.3	113
Antagonists			
Domperidone	0.30 ± 0.03	9.5 ± 0.5	0.032
Haloperidol	0.45 ± 0.03	9.8 ± 0.3	0.046
Spiperone	0.069 ± 0.002	0.61 ± 0.05	0.11
Thiopropazine	0.21 ± 0.01	1.7 ± 0.1	0.12
Prochlorperazine	4.7 ± 0.4	35 ± 3	0.13
(+)Sulpiride	85 ± 7	422 ± 19	0.20
Clozapine	56 ± 2	180 ± 17	0.31
(-)Sulpiride	9.2 ± 0.3	25 ± 1	0.36
Thioridazine	3.3 ± 0.2	7.8 ± 0.8	0.42
Amisulpride	1.7 ± 0.1	3.8 ± 0.3	0.45
Chlorpromazine	2.8 ± 0.2	6.1 ± 0.3	0.46
Raclopride	1.8 ± 0.1	3.5 ± 0.3	0.51
Iodosulpride*	0.61 ± 0.05*	1.2 ± 0.2*	0.52
Pimozide	2.4 ± 0.3	3.7 ± 0.5	0.65
(+)AJ 76	270 ± 14	91 ± 5	3.0
(+)UH 232	40 ± 1	9.2 ± 0.1	4.4

Inhibition constants (K_i) were derived from experiments described in legend of Fig. 2, curves were analysed by computerized nonlinear regression⁴². Means ± s.e.m. of values from 2 or 3 experiments, with closely similar results. The terms agonist and antagonist refer to the actions of the drugs at the D₂ receptor. Compounds are ranked according to their K_{iD2}/K_{iD3} ratios.

* K_i values derived from saturation kinetics at equilibrium.

extrapyramidal symptoms and/or is accompanied by disinhibitory efficacy²⁷. Therefore, these different profiles, detected in clinical and animal behavioural studies^{14,24-26}, might reflect the ability of neuroleptics to differentially block D₂ and D₃ receptors in brain. This hypothesis must be considered with caution, however, in view of the ability of some compounds to interact with non-dopamine receptors, the diversity of recommended therapeutic dosages and interference of pharmacokinetic parameters. The only antagonists exhibiting limited but significantly higher potency at D₃ than at D₂ receptors were UH 232 and AJ 76, two putative autoreceptor selective agents³⁷.

Activation of D₂ receptors in CHO cells by 1 μM dopamine resulted in a 95 ± 2% inhibition of the stimulation of cyclic AMP formation induced by 10 μM forskolin (data not shown), confirming their coupling with a G_i protein³⁸. No such effect was observed on stimulation of D₃ receptors by 1 μM dopamine, and no stimulation of cAMP formation occurred in the absence of forskolin. Hence, unlike D₁ or D₂ receptors, D₃ receptors may not be associated with cAMP formation. This lack of response may however be due to the absence of a suitable G protein or to an inappropriate expression of the D₃ receptor in the recipient cells.

Anatomical distribution

A probe corresponding to the main part of the third intracytoplasmic loop, a domain in which D₂ and D₃ receptors display little homology, was used for hybridizations. A single 8.3-kilobase (kb) band was detected in northern analysis, indicating that the D₃ receptor messenger RNA is among the largest in the superfamily of G protein-coupled receptors; the relative abundance of the message was olfactory tubercle > hypothalamus >

striatum > substantia nigra (Fig. 3a). Autoradiographic pictures obtained after *in situ* hybridization of the D₂ and D₃ receptor mRNAs and labelling both the D₂ and D₃ receptor proteins with [¹²⁵I]iodosulpride are shown in Fig. 4. The distribution of D₃ receptor mRNA partially overlaps but markedly differs from that of D₂ receptor mRNA. For instance, only a weak D₃ receptor hybridization signal was detected in ventral striatum, whereas the whole striatum contains the highest densities of dopamine axons³⁹, D₂ receptor mRNA and [¹²⁵I]iodosulpride binding-sites in the brain. By contrast, the D₃ receptor mRNA is expressed at high levels in the olfactory tubercle-island of Calleja complex and nucleus accumbens. Those areas constitute, with the ventral and ventromedial parts of the caudate putamen, the 'ventral striatum'⁴⁰, a territory receiving afferents from the prefrontal or allocortex, and amygdala and its major dopamine inputs from the A10 cell group in the ventral tegmental area³⁹. This connectivity had led to the designation of this territory as the 'limbic' part of the striatal complex in which D₃ receptors may therefore mediate a large part of dopamine signals. D₂ receptor mRNA is also expressed at high levels in these areas but there is no strict overlap with D₃ receptor mRNA distribution: for instance, the highest levels of D₃ receptor mRNA in brain are detected in the islands of Calleja, in which the D₂ receptor signal is weak, whereas the reverse situation is found in the olfactory tubercles.

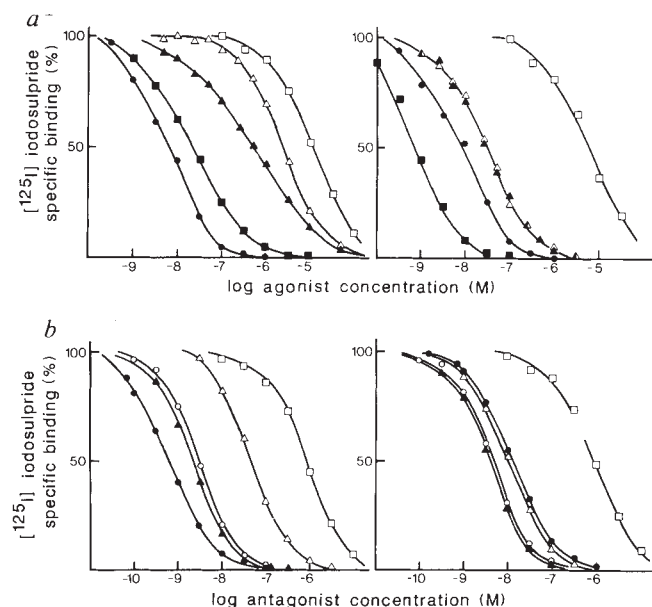


FIG. 2 Compared properties of D₂ (left hand graphs) and D₃ (right hand graphs) receptors expressed in transfected CHO cells. Inhibition of [¹²⁵I]iodosulpride-binding by dopamine agonists (a) and antagonists (b) as follows: □, SKF 38393; △, dopamine + Gpp(NH)p; ▲, dopamine; ■, TL-99; ●, bromocriptine (a) and □, SCH 23390; △, (+)-UH 232; ▲, pimozide; ●, haloperidol; ○, raclopride (b). Agonist and antagonist classification is based on known intrinsic activities at the D₂ receptor and is not necessarily relevant for the D₃ receptor.

METHODS. The expression vectors derive from the pSVβ₂-MDH plasmid used for expression of human β₂ adrenergic receptor⁴⁵ (gift from Dr L. J. Emorine, Institut Pasteur, Paris). The β₂ adrenergic receptor was excised from pSVβ₂-MDH by *Hind*III and *Eco*RI digestions and replaced by a *Sma*I-*Eco*RI restriction fragment of the D_{2A} receptor clone, using a *Hind*III-blunt oligonucleotide adaptor to form the pSVD₂ plasmid. The expression vector for the D₃ receptor was obtained by replacing the *Hind*III-*Bgl*II restriction fragment of pSVD₂ by an *Eco*RI-*Bgl*II restriction fragment of the RT-PCR B clone, using an oligonucleotide adaptor *Hind*III-*Eco*RI to form the pSVD₃ plasmid. These constructs were transfected⁴⁶ to CHO-K1 cells deficient in dihydrofolate reductase and stable transfectants selected in culture medium lacking hypoxanthine and thymidine. Binding experiments were performed¹¹ in 50 mM Tris-HCl buffer containing 120 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂, 0.1% ascorbic acid, 10 μM 8-hydroxyquinoline, 0.1% bovine serum albumin and 0.1 and 0.2 nM [¹²⁵I]iodosulpride in experiments with D₂ and D₃ receptors, respectively.

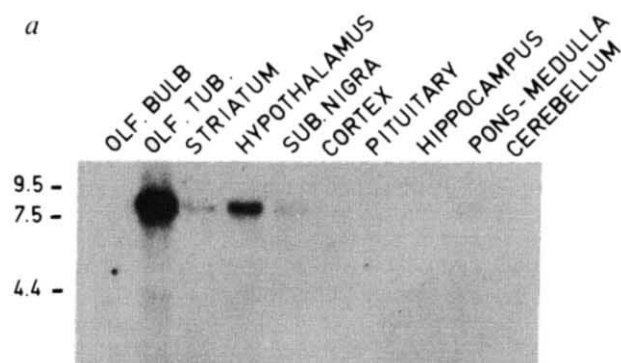


FIG. 3 Characterization of D_3 receptor gene transcripts in various tissues. **a**, RNA blot analysis of D_3 receptor mRNAs from rat brain. Poly(A)⁺ RNAs were prepared^{47,48}. Samples (8 μ g) were denatured, electrophoresed through agarose, transferred to nitrocellulose membranes and hybridized²⁰ using a selective 422-bp 32 P nick-translated *Bam*HI–*Ava*I restriction fragment located within the putative third intracytoplasmic loop (Fig. 1a) at 5×10^6 d.p.m. ml^{-1} . The membranes were exposed for six days at $-80^\circ C$ with intensifying screens. Size of RNA markers (in kb) are shown on the



left. **b**, Autoradiograms of agarose gel electrophoresis of 32 P-labelled cDNA products generated from poly(A)⁺ mRNAs by PCR after 25-cycle (top) and 32-cycle amplification (bottom). Single-stranded cDNA was synthesized using 1 μ g RNA, 20 U AMV reverse transcriptase and primer 3 (Fig. 1a). Double-stranded cDNAs were synthesized and amplified using 2.5 U *Taq* polymerase, primer 3 and an oligonucleotide derived from amino acids 300–306 (Fig. 1b): 5'-AAGCGCTACTACAGCATCTGC (primer 5) for 25 or 32 cycles ($92^\circ C$, $56^\circ C$ and $72^\circ C$, 1 min each). A tracer dose of (32 P)dCTP was incorporated during the amplification and DNA electrophoresed in 1.5% agarose. PREF. CORTEX, prefrontal cortex; OLF. BULB, olfactory bulb; OLF. TUB, olfactory tubercle; SUB. NIGRA, substantia nigra; VTA, ventral tegmental area.

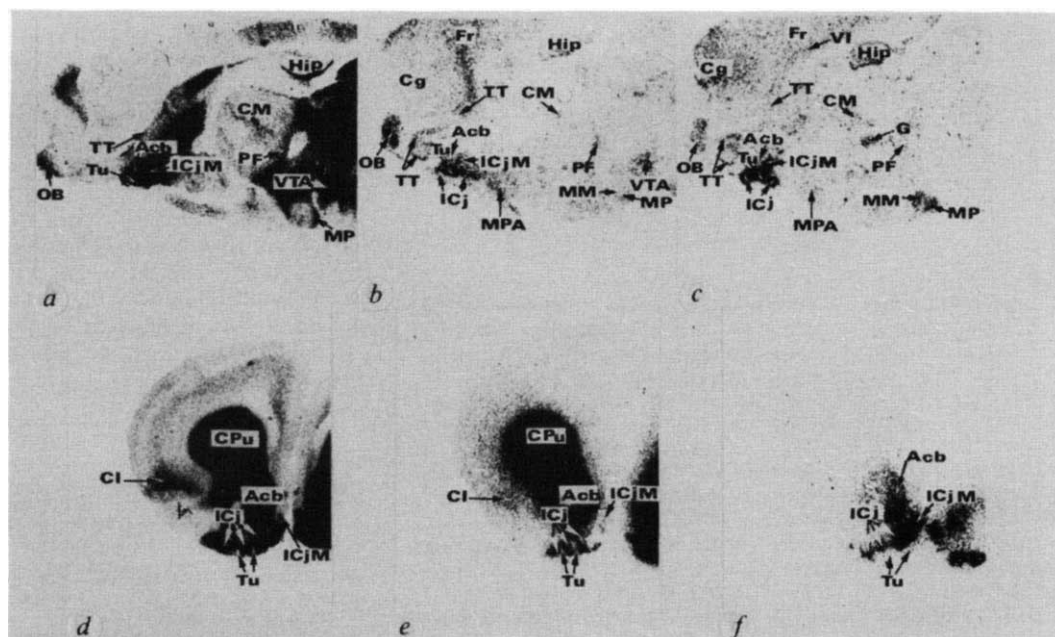
In this part of the striatal complex, the distribution of D_3 receptor signals seems to overlap that of limbic and allocortical projections, whereas the remainder of the striatal complex, which is mainly innervated by dopamine projections from the substantia nigra, receives its cortical inputs from the somatic neocortex³⁹ and is rich in D_2 receptors. This suggests an important participation of D_3 receptors in dopaminergic transmissions in limbic areas known to be associated with cognitive, emotional and endocrine functions. D_3 receptor signals were also detected in other 'limbic' areas such as the hippocampus, septum or mammillary nuclei in the hypothalamus.

No D_3 receptor signal could be detected in the pituitary—a prototypic location for D_2 receptors—even using PCR. This suggests that selective D_3 receptor ligands, when available in therapeutics, unlike the currently used neuroleptics, will not affect the activity of mammothrophs. Finally, a D_3 receptor signal was detected in kidney, an organ in which dopamine in low dosage exerts vasodilatory actions.

The D_3 receptor as a second autoreceptor

Both [125 I]iodosulpride binding sites⁴¹ and D_2 receptor mRNAs are abundant over dopamine perikarya in the ventral tegmental

FIG. 4 Autoradiographic localization of [125 I]iodosulpride binding sites and D_2 or D_3 receptor mRNAs in rat brain. Sagittal (**a, b, c**) or coronal (**d, e, f**) sections (laterality 0.4 mm or interaural level 10.7 mm⁴⁹) were incubated with either [125 I]iodosulpride (**a, d**), a probe for both D_2 and D_3 receptors, or with 32 P-labelled RNA probes selective for D_2 receptor (**b, e**) or D_3 receptor mRNAs (**c, f**). Abbreviations: Acb, accumbens nucleus; Cl, claustrum; Cg, cingulate cortex; CM, central medial thalamic nucleus; CPu, caudate putamen; Fr, frontal cortex; G, gelatinous thalamic nucleus; Hip, hippocampal formation; ICj, islands of Calleja; ICjM, major island of Calleja; MM, medial mammillary nucleus medial part; MP, medial mammillary nucleus posterior part; MPA, medial preoptic area; OB, olfactory bulb; PF, parafascicular thalamic nucleus; Tu, olfactory tubercle; TT, tenia tecta; VTA, ventral tegmental area; VI, layer VI of cerebral cortex.



METHODS. [125 I]iodosulpride binding was performed on unfixed cryostat sections incubated with 0.3 nM [125 I]iodosulpride, rinsed and apposed for 5 days to 3 H-Hyperfilm (Amersham)⁴¹. For *in situ* hybridization studies, 32 P-labelled single-stranded RNA probes were synthesized using a Riboprobe kit (Promega) with [32 P]UTP and T7 RNA polymerase. Templates (10 ng)

were *Eco*RI-digested pGEM-4Z recombinant plasmids containing a *Sac*I–*Afl*II fragment of D_2 receptor clone or a *Bam*HI–*Ava*I fragment of RT-PCR B clone in the proper orientation. Cryostat sections (10 μ m) were mounted on slides, fixed in 4% formaldehyde, treated with proteinase K and hybridized overnight at $55^\circ C$ with 32 P-labelled probes at 2.10^6 d.p.m. in 50 μ l. Slides were treated with RNase A, washed in $0.5 \times$ SSC buffer at $55^\circ C$ according to Meadoz-Woodruff *et al.*⁵⁰, dehydrated and apposed to β max Hyperfilms (Amersham) for 20 days at $-80^\circ C$.

area and substantia nigra, but D_3 receptor signals could not be detected by *in situ* hybridization (Fig. 4). However, clear signals were detected by northern and PCR analysis (Fig. 3), and the hypothesis that D_3 receptors are expressed by dopamine neurons themselves was verified after lesioning these areas with 6-hydroxydopamine, a toxin that selectively ablates catecholaminergic neurons (Fig. 5). After degeneration of these neurons, we found a marked ipsilateral reduction of the PCR-generated signal for the D_3 receptor in both the substantia nigra ($-65 \pm 10\%$) and the ventral tegmental area ($-69 \pm 14\%$). In the same tissue extracts, D_2 receptor mRNA levels were similarly affected, that is, by -88% and -65% , respectively.

This established that both D_2 and D_3 receptors are expressed by dopamine neurons belonging to the A_9 and A_{10} cell groups, and suggests that both act as autoreceptors. Such a role for the D_3 receptor is consistent with its high apparent affinity for dopamine, as this amine in very low concentrations, reduces the electrical activity of dopaminergic neurons^{17,18}. This is supported by the pharmacological profile of the D_3 receptor, at which the most discriminant agonists and antagonists were previously categorized as autoreceptor-selective agents in electrophysiological, biochemical or behavioural studies¹⁷.

Many distinct functions were previously attributed to dopamine autoreceptors, such as inhibitions of impulse flow, dopamine synthesis and release at either nerve terminals or dendrites, and co-transmitter release^{17,18}. D_2 and D_3 autoreceptors might variously participate in all these actions in various brain areas. Finally the question as to whether a single

cell expresses both D_2 and D_3 receptors remains to be answered by *in situ* hybridization studies at the cellular level.

Discussion

The D_3 receptor differs from the previously defined D_1 and D_2 receptors in its molecular properties, pharmacology, tissue distribution and, presumably, transduction system. In addition, it is mainly expressed in discrete brain areas belonging or related to the limbic system, whereas D_1 and D_2 receptors are widely expressed in all major dopaminergic areas. The D_3 receptor, like the D_2 receptor, seems to be both an autoreceptor and a postsynaptic receptor. But the most important feature with regard to therapeutic considerations is that the D_3 receptor seems to be a molecular target for drugs currently used to alleviate major neurological or psychiatric symptoms, that were thought previously to act specifically through interaction with a single receptor, the D_2 receptor.

The characterization of the D_3 receptor may account for some previously unexplained observations and will allow new therapeutic developments. For instance, many biochemical or behavioural studies using purported D_2 -selective receptor ligands and showing pluriphasic responses according to the dose, might be reinterpreted, taking into account the interaction of these drugs with both pre- and postsynaptic D_2 and D_3 receptors in various brain areas. More importantly, activation and blockade of both D_2 and D_3 receptors presumably take place in patients with Parkinson's disease treated with dopamine agonists and in schizophrenics treated with neuroleptics, respectively. This dual interaction of drugs might partly account for their distinct clinical profiles and side-effects; for example, the similar affinities of atypical neuroleptics at D_2 and D_3 receptors may explain their disinhibitory efficacy and/or lesser propensity to cause extrapyramidal side-effects. If confirmed, such comparison should allow predictions, by simple screening tests, of the clinical profiles of drugs that were previously discovered by serendipity or selected by behavioural tests in animals.

The present study does not so far answer the important question as to whether the blockade of the D_2 or D_3 receptor (or of both) is responsible for antipsychotic activity. The high affinity of most neuroleptics for the D_3 receptor, the selective expression of this receptor in limbic areas controlling cognitive functions and emotional behaviour, would be consistent with the predominant role of this receptor. But the marked preference of haloperidol for the D_2 receptor designates this as the major receptor to be blocked in eliciting antipsychotic activity; however, the haloperidol concentrations in the plasma of successfully treated schizophrenics²⁷ do not exclude the possibility that both D_2 and D_3 receptors might be blocked in the brain. The molecular cloning of the D_2 and D_3 receptors should facilitate the design of more discriminant drugs to evaluate these possibilities. Such drugs may constitute safer and more effective therapeutic agents in psychiatry and neurology. □

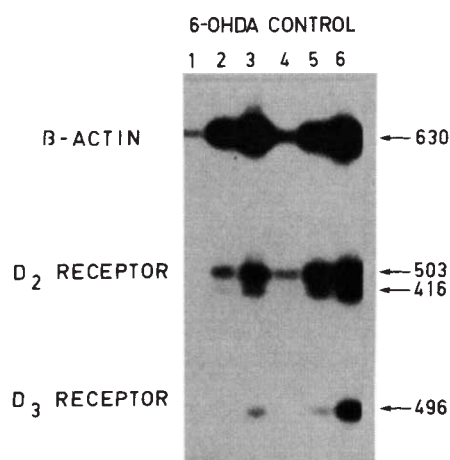


FIG. 5 Effects of 6-hydroxydopamine-induced lesions on the levels of D_2 and D_3 receptor gene transcripts in the substantia nigra. Rats received a unilateral injection of 6-hydroxydopamine ($8 \mu\text{g}$ per $4 \mu\text{l}$ saline) in the medial forebrain bundle at coordinates A 4.7 mm, L 1.5 mm and H 1.0 mm⁴⁹. After 10 days, rats displaying contralateral rotations after administration of 0.25 mg kg^{-1} apomorphine were selected. They were killed 4–7 weeks after lesion and total RNA from individual substantia nigra or ventral tegmental area was prepared⁵¹. PCR experiments were as described in Fig. 3 using oligonucleotides derived from amino acids 43–51: 5'-GATGGTGGGTATGGGTCAGAAGGA and amino acids 243–251: 5'-GCTCATTGCCGATAGTGATGACCT for β actin. For the D_2 receptor, oligonucleotides derived from amino acids 195–205: 5'-TCCGAATTCTCATTCTACGTGCCCTTCATC and amino acids 355–363: 5'-GCTTTCTGCGGCTCATCGTCTTAA were used. Primers 3 and 5 (Fig. 3) were used for the D_3 receptor. PCR products obtained after 16 (lanes 1 and 4), 23 (lanes 2 and 5) and 27 cycles (lanes 3 and 6) for β actin and after 23 (lanes 1 and 4) 27 (lanes 2 and 5) and 30 cycles (lanes 3 and 6) for D_2 and D_3 receptors with the substantia nigra of a typical rat (lanes 1–3, lesion side; lanes 4–6, control side). The experiment was repeated with individual tissues of 4–7 rats and parallel experiments were conducted with the ventral tegmental area. Gel autoradiograms, corresponding to 23 and 30 cycles for β -actin and receptors respectively, were scanned. The ratio of optical densities corresponding to receptors and β -actin was calculated. Ratios obtained for the lesion side were then compared to ratios for the control side in each animal. DNA size markers (bp), right hand side.

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Identification of the 64K autoantigen in insulin-dependent diabetes as the GABA-synthesizing enzyme glutamic acid decarboxylase

Steinunn Baekkeskov*, Henk-Jan Aanstoot*, Stephan Christgau*, Annette Reetz†, Michele Solimena†, Marilia Cascalho*, Franco Folli†‡, Hanne Richter-Olesen|| & Pietro-De Camilli*

* Departments of Microbiology/Immunology, Medicine, and Hormone Research Institute, University of California San Francisco School of Medicine, San Francisco, California 94143, USA

† Department of Cell Biology, Yale University School of Medicine, New Haven, Connecticut 06510, USA

‡ Present address: Department of Medicine, Istituto Scientifico S. Raffaele, Milano, Italy

|| Hagedorn Research Laboratory, DK2820 Gentofte, Denmark

The pancreatic islet β -cell autoantigen of relative molecular mass 64,000 (64K), which is a major target of autoantibodies associated with the development of insulin-dependent diabetes mellitus (IDDM) has been identified as glutamic acid decarboxylase, the biosynthesizing enzyme of the inhibitory neurotransmitter GABA (γ -aminobutyric acid). Pancreatic β cells and a subpopulation of central nervous system neurons express high levels of this enzyme. Autoantibodies against glutamic acid decarboxylase with a higher titre and increased epitope recognition compared with those usually associated with IDDM are found in stiff-man syndrome, a rare neurological disorder characterized by a high coincidence with IDDM.

THE cell-specific destruction of pancreatic β cells, which precedes the clinical onset of insulin-dependent diabetes mellitus is believed to be mediated by autoimmune mechanisms¹. The autoimmune phenomena associated with the disease include massive lymphocytic infiltration of islets² and circulating

autoantibodies to β cells³. A 64K β -cell autoantigen is a target of autoantibodies in this disease⁴. The 64K autoantibodies are present in $\geq 80\%$ of newly diagnosed IDDM patients and have been detected up to several years before clinical onset of IDDM concomitant with a gradual loss of β cells⁵⁻⁷. The 64K antigen was found to be β cell-specific in an analysis of several tissues which did not include the brain⁸. The 64K autoantigen in β cells is detected as a hydrophilic soluble 65K form and a 64K hydrophobic form which can be both membrane bound and soluble (H. Schierbeck, L. Aagaard and S.B., manuscript submitted). The 64K forms can be resolved into two components, α and β (ref. 9). The function of the 64K protein in the β cell has remained elusive.

Most patients with a rare but severe neurological disease called stiff-man syndrome (SMS) have autoantibodies to GABA-secreting neurons. Glutamic acid decarboxylase (GAD), the enzyme that synthesizes GABA from glutamic acid, is the predominant autoantigen^{10,11}. Surprisingly, almost all patients positive for the autoantibody to GABA-secreting neurons are also positive for islet cell cytoplasmic antibodies, as demonstrated by immunofluorescence of pancreatic sections, and a significant fraction have IDDM¹¹. GAD is selectively expressed in GABA-secreting neurons in the central nervous system (CNS)¹². Outside neurons, GAD is found at high levels in pancreatic β cells¹³⁻¹⁵. There are at least two isomers of GAD in brain, which are resolved by differences in their mobility on SDS-polyacrylamide

gel electrophoresis; their molecular weights have been described as 59–66K (ref. 16).

Because IDDM is the autoimmune disease that is most often associated with SMS and because some of the characteristics of GAD and the 64K autoantigen are similar, we hypothesized that they were the same protein. Here we demonstrate that the 64K autoantigen in IDDM is the same as GAD in pancreatic β cells.

Immunoprecipitation of 64K autoantigen

We first assessed whether serum S3, a sheep antiserum raised against purified rat brain GAD¹⁷, and sera from SMS patients positive for GAD antibodies^{10,11}, could immunoprecipitate the 64K autoantigen from rat islets. We used [³⁵S]methionine-labelled rat islet cell fractions partially enriched for the 64K antigen (S-100 DP, Fig. 1). The sera positive for GAD antibody (anti-GAD sera) immunoprecipitated a doublet of [³⁵S]methionine-labelled proteins, which on SDS-PAGE have the same mobility as the 64K α/β autoantigen immunoprecipitated by IDDM sera⁷ (Fig. 1a, lanes 4–9).

To assess whether the proteins recognized by the anti-64K-sera and by anti-GAD sera were the same, supernatants resulting from immunoprecipitation with anti-GAD sera were subsequently reprecipitated with anti-64K IDDM sera. Similarly, supernatants resulting from immunoprecipitation with anti-64K IDDM sera were reprecipitated with anti-GAD sera. Results from those experiments showed that anti-GAD sera, and anti-64K sera each quantitatively removed the protein recognized by the other group of sera, demonstrating complete cross-reactivity between anti-GAD sera and anti-64K sera, and strongly suggesting that the 64K protein is the same as

GAD in rat islets (Fig. 1a, lanes 10–20).

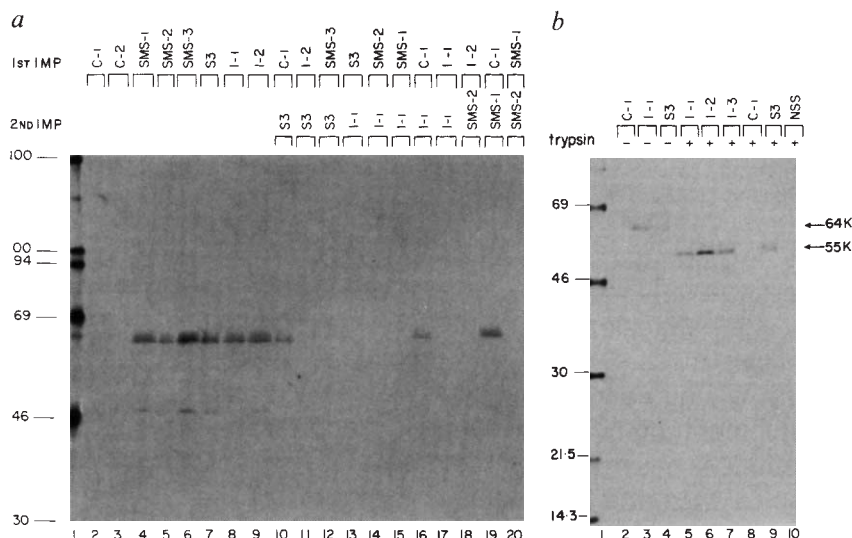
Trypsin digestion of [³⁵S]methionine-labelled islet cell extracts followed by immunoprecipitation showed that a 55K immunoreactive fragment was formed from both the 64K protein and GAD, further verifying their common identity (Fig. 1b). Furthermore, two-dimensional analyses of GAD immunoprecipitated from [³⁵S]methionine-labelled islets with the S3 antiserum revealed an identical pattern to that described for the 64K protein (H. Schierbeck, L. Aagaard and S.B., manuscript submitted; and ref. 9) (results not shown). This pattern was also the same as the two-dimensional pattern of pancreatic and brain GAD shown by western blotting of two-dimensional gels with the S3 serum (see Fig. 5a).

Immunoprecipitation of GAD

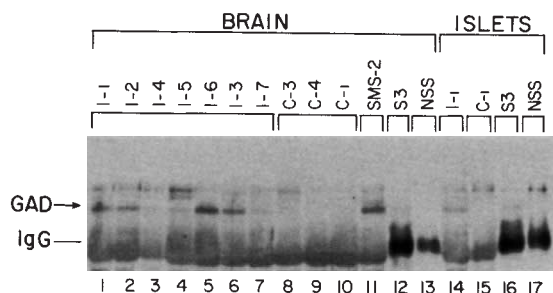
We next analysed whether anti-64K sera could immunoprecipitate GAD from brain and islets. Membrane and soluble fractions were prepared from brain and islets and immunoprecipitated with serum S3, anti-GAD SMS sera, anti-64K IDDM sera, and control sera. Presence of GAD in the immunoprecipitates was analysed by western blotting. The blots were probed with serum S3 or serum 7673, a rabbit serum raised to a synthetic 17-amino-acid peptide corresponding to the carboxyl terminus of the larger rat brain GAD isoform (ref. 18; and D. Gottlieb, personal communication) (M.S. and A.R., unpublished data). Both sera gave identical results. Figure 2 shows a western blot containing some of such immunoprecipitates probed with serum S3. As expected, an immunoreactive band with the electrophoretic mobility of GAD was detected in immunoprecipitates obtained with anti-GAD sera (Fig. 2, lanes 11, 12, 16). A band of identical mobility was visualized in all immunoprecipitates obtained with

FIG. 1 Anti-GAD sera and anti-64K IDDM sera recognize the same protein in rat islets. **a**, Fluorogram of an SDS-PAGE showing immunoprecipitation of Triton X-114 detergent phase cytosolic fraction from [³⁵S]methionine-labelled rat islets (S-100 DP) with anti-GAD sera, anti-64K IDDM sera and control sera. Lanes 2–9, samples from a single immunoprecipitation with sera indicated at the top of each lane; lanes 10–20, samples from a second immunoprecipitation of supernatants remaining after a first immunoprecipitation. Sera used for the first and second immunoprecipitations are indicated at the top of each lane. Sera used for the immunoprecipitation were: C, sera from healthy individuals; SMS, sera of SMS patients previously shown to be GAD antibody positive¹¹; I, sera from newly diagnosed anti-64K-positive IDDM patients⁷ (numbers indicate patient code); S3, a sheep antiserum raised to purified rat brain GAD¹⁷. Relative molecular mass markers are shown in lane 1 ($M_r \times 10^{-3}$). The anti-GAD sera immunoprecipitate GAD from supernatants after immunoprecipitation with control serum (lanes 10 and 19), but not from supernatants after immunoprecipitation with anti-64K sera (lanes 11 and 18). The anti-64K sera immunoprecipitate the 64K protein from supernatants after immunoprecipitation with control serum (lane 16), but not from supernatants after immunoprecipitation with anti-GAD sera (lanes 13–15). The triple band represents the 65K form and 64K α and β forms of the 64K autoantigen (H. Schierbeck, L. Aagaard and S.B., manuscript submitted). **b**, Fluorogram showing immunoprecipitation of the 64K protein and GAD from S-100 DP of [³⁵S]methionine-labelled rat islets before (lanes 2–4) and after (lanes 4–10) trypsin digestion. Serum codes are as in legend for **a**. NSS is a preimmune sheep serum. Trypsin digestion results in a 55K immunoreactive fragment that is recognized by both anti-64K sera and the anti-GAD serum S3.

METHODS. Neonatal rat islets were isolated and labelled with [³⁵S]methionine as described⁹. Islets were swollen on ice for 10 min in 10 mM HEPES, pH 7.4, 1 mM MgCl₂ and 1 mM EGTA (HME buffer) and then homogenized by 20 strokes in a glass homogenizer. The homogenate was centrifuged at 2,000g to remove cell debris and the postnuclear supernatant centrifuged at 100,000g for 1 h to obtain a cytosol (S-100) and a particulate (P-100)



fraction. Amphiphilic proteins were purified from the S-100 fraction by a modification of the method described by Bordier²⁹ for Triton X-114 (TX-114) phase separation. S-100 fraction was made 1% in TX-114, warmed at 37 °C for 2 min to induce TX-114 phase transition, and centrifuged at 15,000g for 2 min to separate the aqueous and detergent phases. The detergent phase was diluted in 20 mM Tris buffer, pH 7.4, 150 mM NaCl (TBS) and immunoprecipitated as described⁹ using DP from 300 islets for each immunoprecipitate. Immunoprecipitates were analysed by SDS-PAGE (10%) and processed for fluorography²⁴. For the trypsin digestion, S-100 DP from 4,000 islets was diluted to 200 μ l in TBS. Trypsin (0.74 units) was added and the sample was incubated for 1 h at 25 °C. The reaction was stopped by addition of 10 μ l 10 mM HEPES, 5 mM EDTA, 5 mM pyrophosphate, 5 mM benzamidine-HCl, pH 7.5. Digested and undigested material was immunoprecipitated and the immunoprecipitates analysed by SDS-PAGE (15%). Anti-64K sera were from three newly diagnosed IDDM patients⁷, control sera were from two healthy individuals. SMS sera were from three anti-GAD-positive individuals¹¹. The S3 antiserum was a gift from I. J. Kopin.



anti-64K sera (7/7) (Fig. 2, lanes 1-7, 14), but not in those obtained with control sera (Fig. 2, lanes 8-10, 13, 15, 17). These results demonstrate that the protein immunoprecipitated from brain and islets by anti-64K sera is indistinguishable from GAD.

64K protein has GAD enzyme activity

If the 64K autoantigen is GAD, then the 64K protein should have the enzymatic properties of GAD. We therefore investigated whether GAD enzyme activity could be removed from islet and brain cell lysates by immunoprecipitation with an anti-64K IDDM serum, and whether GAD activity could then be measured in the immunoprecipitates. Brain and islet cell fractions were immunoprecipitated with increasing amounts of an anti-64K IDDM serum, and the GAD enzyme activity measured after immunoprecipitation in both supernatants and pellets (Fig. 3a, b). Immunoprecipitation with increasing amounts of anti-64K serum but not with control serum removed

FIG. 2 Anti-64K antibodies immunoprecipitate GAD from brain and islets. Western blot of immunoprecipitates of rat brain (S-100 DP) and islet cell (P-100 DP) fractions obtained with anti-64K sera and anti-GAD sera. The blot was probed with the S3 serum. Sera used for the immunoprecipitation are indicated at the top of each lane by the same codes as in Fig. 1. The 64K protein immunoprecipitated from both brain and islets is immunostained by anti-GAD antibodies. In the gel shown GAD migrated as a single band. METHODS. Neonatal rat brain was homogenized at 4 °C in seven volumes of HME buffer followed by centrifugation at 100,000g for 1 h to obtain S-100 and P-100 fractions. S-100 DP was prepared and aliquots (1/13 brain per lane) immunoprecipitated as described in the legend to Fig. 1. P-100 was prepared from neonatal rat islets and extracted in 200 ml TBS with 1% Triton X-114 for 2 h at 4 °C (ref. 9). P-100 DP was prepared as described for S-100 DP and aliquots (1,500 islets per lane) immunoprecipitated. Immunoprecipitates were subjected to SDS-PAGE followed by electroblotting to a PVDF membrane (Immobilon)³⁰, probing with the S3 serum and visualizing by alkaline phosphatase-conjugated rabbit anti-sheep IgG.

the GAD activity from both brain and islet cell lysates in a dose-dependent manner, and, in parallel, increasing amounts of GAD activity appeared in the immunoprecipitates. The GAD activity recovered in the immunoprecipitates did not account for all the activity lost from the supernatants, probably owing to an inhibiting effect of antibodies on enzyme activity. For islet cell extracts from [³⁵S]methionine-labelled islets, SDS-PAGE revealed that the only islet cell protein specifically detected in the immunoprecipitates obtained with the anti-64K IDDM serum was the 64K autoantigen (see Fig. 1). Thus the GAD enzyme activity measured in immunoprecipitates obtained with the anti-64K IDDM serum is a property of the 64K autoantigen.

Brain and β -cell GAD

Analysis of GAD enzyme activity in neonatal and adult rat tissues showed that the expression of GAD is high in brain and islet cells and is either absent or low in a variety of other tissues (results not shown), confirming previous reports¹⁹. In islets,

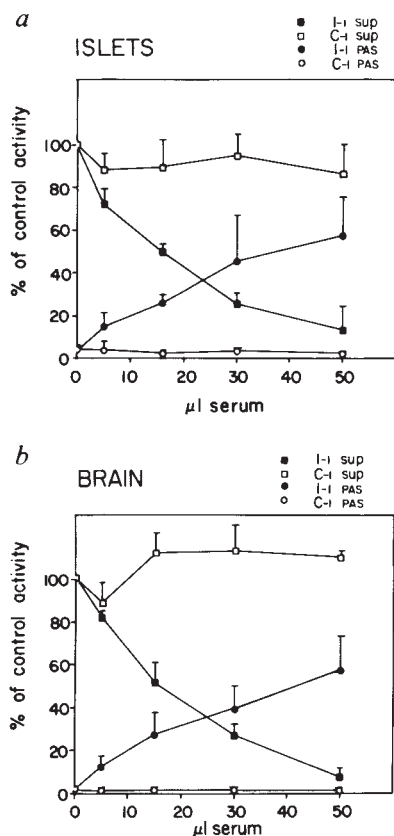


FIG. 3 Precipitation of GAD activity from brain and islets with anti-64K autoantibodies. a, Aliquots (700 islets per sample) of S-100 DP from neonatal rat islets were immunoprecipitated with increasing amounts of the anti-64K IDDM serum I-1 (closed symbols) or the control serum C-1 (open symbols). GAD activity was measured in supernatants after immunoprecipitation (squares) and in pellets (circles). The activity in the supernatants was calculated as percentage of the activity in non-immunoprecipitated samples incubated with the same amount of control serum. The activity in the immunoprecipitates was calculated as percentage of the activity in samples incubated without serum. Values are mean of three experiments $\pm$ s.d. b, As a, except that aliquots of S-100 DP from neonatal rat brain were used instead of islet cell material.

METHODS. S-100 DP was prepared from islets and brain as described in the legends to Figs 1 and 2, except that buffers were supplemented with 1 mM aminoethylisothiuronium bromide hydrobromide (AET) and 0.2 mM pyridoxal 5'-phosphate (PLP). S-100 DP was diluted 10 times in 50 mM potassium phosphate pH 6.8, 1 mM AET, 0.2 mM PLP (buffer A) and incubated with the indicated amounts of sera in a total volume of 150 μ l for 7 h at 4 °C. Immunocomplexes were absorbed to 150 μ l protein A-Sepharose beads (PAS Pharmacia) and isolated by centrifugation. The supernatants were collected and centrifuged three times to remove traces of PAS. PAS pellets were washed five times by centrifugation in buffer A. Enzyme activity in the PAS pellets and the supernatants was measured using a modified version of the assay first described by Albers and O'Brady³¹. Both PAS pellets and supernatants were transferred to 1.5-ml screw cap tubes; 20 μ l 5 mM L-glutamate in buffer A and 0.4 μ Ci [¹⁴C]L-glutamate (59 mCi mmol^{-1} , Amersham) were added. The tubes were closed immediately with a cap containing Whatman filter paper soaked in 50 μ l 1 M hyamine hydroxide in methanol and incubated for 2 h at 37 °C. The filter paper was then removed and the absorbed ¹⁴CO₂ measured in a scintillation counter. The specific activity of GAD in homogenates of neonatal brain and islet cell material was similar (~40-70 mU per g protein).

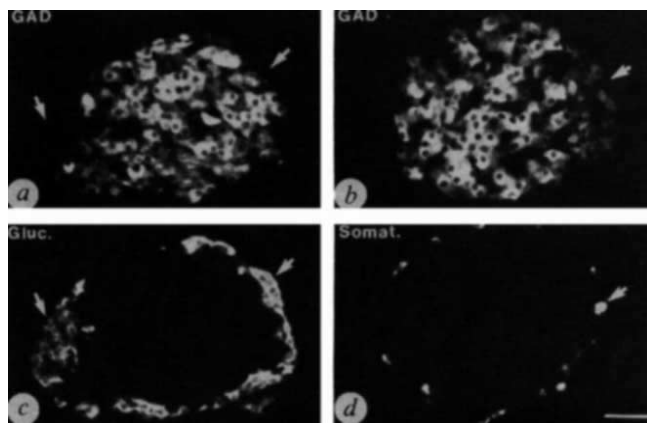


FIG. 4 Immunofluorescence staining of pancreatic islets with GAD, glucagon and somatostatin antibodies. Immunofluorescence micrograph showing pancreatic islets. The islet in *a* and *c* was double-labelled for GAD and glucagon. The islet in *b* and *d* was double-labelled for GAD and somatostatin. Arrows point to corresponding cells in the two pairs of panels. The β cells in the central core of the islet are brightly stained with the GAD antibody, whereas the cells positive for glucagon and somatostatin do not stain with the same antibody.

METHODS. Formaldehyde-fixed frozen sections of rat pancreas were first rhodamine-labelled for GAD using a mouse monoclonal GAD6, raised against purified rat brain GAD¹⁶, and then fluorescein-labelled for glucagon or somatostatin using rabbit polyclonal antibodies to either hormone and using methods described³². GAD6 was a gift from D. I. Gottlieb, University of Washington. Scale bar, 25 μ m.

double immunostaining with a monoclonal antibody to GAD and either glucagon or somatostatin confirmed the localization of GAD to the β -cell core, and the absence of GAD in the other endocrine cells, which are localized to the islet periphery (Fig. 4). GAD in brain and islets was found to have identical mobility on SDS-PAGE (Fig. 2) and by two-dimensional gel electrophoresis using isoelectric focusing/SDS-PAGE (Fig. 5*a*). We compared the immunoreactive trypsin fragments generated from brain and islet GAD. Trypsin generated a 55K immunoreactive fragment from both islet and brain GAD (Fig. 5*b*; see also Fig. 1). In both tissues GAD was found in a soluble hydrophobic form as well as a membrane-bound hydrophobic form (Fig. 5*b*) as described for the 64K islet cell autoantigen (H. Schierbeck, L. Aagaard and S.B., manuscript submitted). The 65K component of the 64K protein in islets (H. Schierbeck, L. Aagaard and S.B., manuscript submitted) was detected in brain and islet cells in some (Figs 1*a*, 5*A*, *b*, *c*, 5*B* and 6*A*) but not in other analyses (Figs 1*b*, 2). Furthermore, the 64K α/β doublet was detected in some analyses (Figs 1*a*, 6*A*). The immunochemical

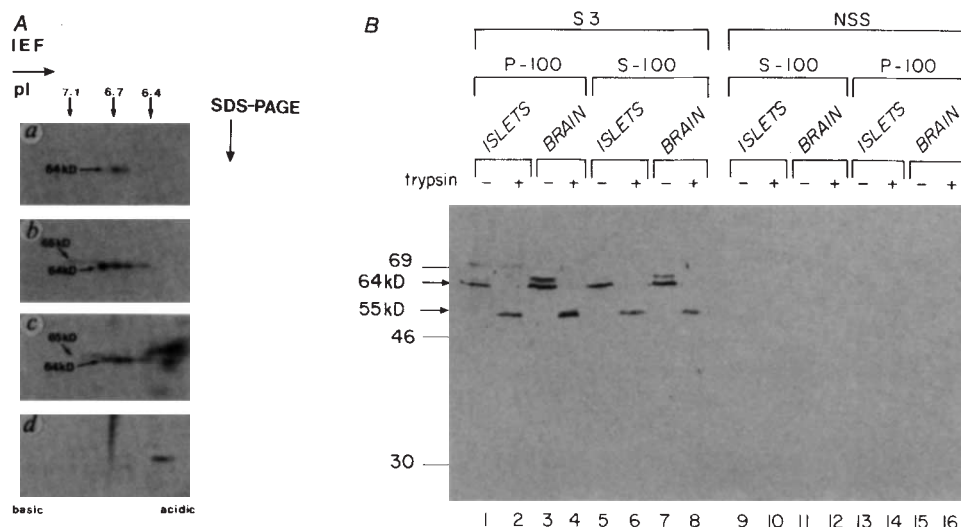
and biochemical properties of the brain and islet GAD therefore indicate that they are very similar.

Antibodies to GAD in SMS and IDDM sera

The GAD reactivity in SMS sera has been demonstrated by western blotting and by immunocytochemical staining of fixed tissue sections^{10,11}, that is, assays that involve complete or partial denaturation of the antigen. The standard assay for 64K antibodies in IDDM sera has been immunoprecipitation from islet cell lysates prepared in nondenaturing conditions⁴⁻⁹. We selected sera from the individuals who had the highest immunoreactivity to the 64K autoantigen in immunoprecipitation experiments in a survey of 112 IDDM patients and prediabetic individuals without SMS⁷ and tested them for immunoreactivity to the brain GAD protein on western blots (five sera) and for immunostaining of GABA-secreting neurons (7 sera). The results were compared with those for SMS sera. In contrast to the SMS sera, none of the IDDM sera detected the denatured GAD protein on western blots (Fig. 6*A*), and only one was able

FIG. 5 GAD in brain and islets have similar properties. **A**, Two-dimensional gel electrophoresis of neonatal rat and islet cell GAD/64K antigen. *a*, *b*, Western blots of two-dimensional gels of a neonatal islet particulate fraction (*a*) and a brain fraction (*b*) probed with the GAD anti-serum S3. *c*, *d*, Fluorograms of two-dimensional gels of immunoprecipitates of a [³⁵S]methionine-labelled rat islets extract (S-100 DP) with the anti-64K IDDM serum I-1 (*c*) and *a* with a control serum C-1 (*d*). The soluble fractions of brain and islets (*b* and *c*) contain both the 65K pI 7.1 component and the 64K 6.7 α component, whereas the particulate fraction contains only the 64K pI 6.7 α component (H. Schierbeck, L. Aagaard and S.B., manuscript submitted). Both the 65K and the 64K component display charge heterogeneity previously described for the 64K autoantigen in islets⁹. The panels both demonstrate the identical behaviour of the 64K protein (*c*) and GAD (*a* and *b*), further proving they are the same protein, and show the similarities of brain and islet GAD with regard to both charge and size. **B**, Western blot of soluble and particulate GAD from neonatal rat brain and islets before and after trypsin digestion. Lanes 1–8, probing with S3 serum; lanes 9–16, probing with normal (preimmune) sheet serum. Trypsin digestion of both brain and islet GAD results in a 55K immunoreactive GAD fragment.

METHODS. A particulate fraction was prepared from neonatal rat islet homogenates by centrifugation at 36,000*g* (*a*). A low-speed synaptosomal supernatant was prepared from brain as described³³ (*b*). S-100 DP was



prepared from neonatal rat islets and immunoprecipitated as described in the legend to Fig. 1 (*c* and *d*). Two-dimensional gel electrophoresis was performed as described by O'Farrell³⁴ and modified by Ames and Nikaido³⁵. Immunoblotting was according to Towbin *et al.*³⁰. GAD in *a* and *b* was visualized by probing with the anti-GAD serum S3, followed by rabbit anti-sheep IgG serum and ¹²⁵I-labelled protein A and autoradiography. For *B*, S-100 DP and P-100 DP from islets and brain were prepared as described in legends to Figs 1 and 2 and digested with trypsin as described in legend to Fig. 1. Brain and islet cell fractions were subjected to SDS-PAGE using 15% polyacrylamide gel. Western blotting and staining procedures were as described in legend to Fig. 2.

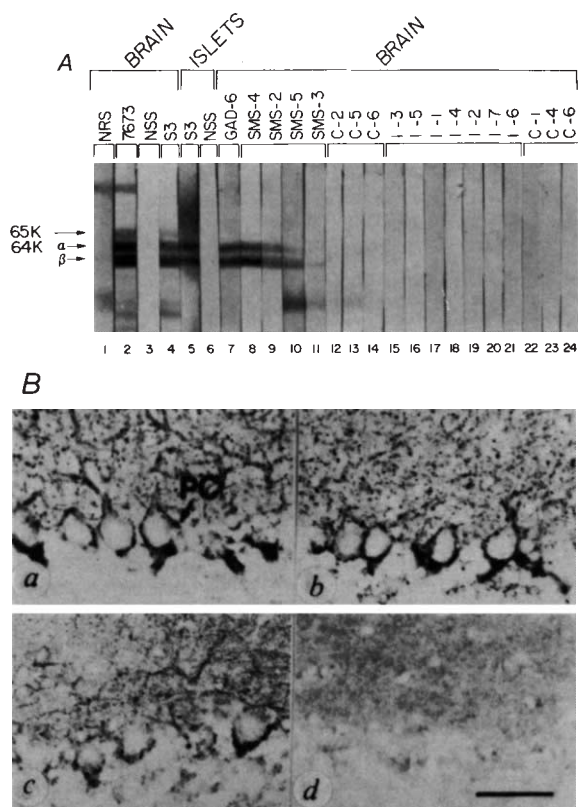


FIG. 6 Comparison of GAD immunoreactivity in SMS and IDDM sera. *A*, Western blots of S-100 DP from neonatal rat brain (lanes 1–4 and 7–24) and islets (lanes 5 and 6) probed with anti-GAD SMS sera and sera from IDDM patients and prediabetic individuals with high immunoreactivity to the 64K/GAD protein in immunoprecipitation experiments. Sera are indicated at the top of each lane by the same codes as in Fig. 1. The S3 serum, 7673 serum, GAD6 serum and anti-GAD SMS sera recognize the denatured form of GAD in brain and islets, whereas the anti-64K IDDM sera do not. The S3 and 7673 sera react with both the 65K form and the 64K α and β forms, whereas GAD6 (previously shown to react selectively with the smaller brain GAD isoform¹⁶) and the SMS sera only react with the 64K α and β components. *B*, Bright-field light microscopy micrographs showing immunostaining of GABA-secreting nerve terminals in the rat cerebellar cortex with SMS and IDDM sera. *a*, SMS-3 serum; *b*, IDDM serum I-8; *c*, IDDM serum I-2; *d*, IDDM serum I-1. All sera, except I-1, show the typical stain of GABA-secreting nerve terminals. Note the accumulation of immunoreactivity at the base of the Purkinje cells, where the GABA-secreting nerve endings of basket cells terminate.

METHODS. S-100 DP aliquots from rat brain and rat islets were subjected to SDS-PAGE, electroblotted and immunostained as described in the legend to Fig. 2. Dilutions for immunostaining of western blots were 1/200 for GAD6, 1/250 for IDDM sera, control human sera, SMS sera 2 and 5, the 7673 serum and normal rabbit serum, 1/500 for SMS sera 3 and 4 and 1/2,000 for S3 and normal (preimmune) sheep serum. For *B*, methods as described in ref. 11.

to weakly immunostain GABA-secreting neurons (Fig. 6*B*, *c*). Titration of IDDM and SMS sera in immunoprecipitation experiments furthermore showed that the titre of anti-GAD antibodies in SMS sera was 10–200 times that in IDDM sera (results not shown). In another survey of 74 IDDM patients, only three were positive for antibodies to GABA-secreting neurons (Fig. 6*B*, *b*) including the only two which were positive by western blotting (ref. 11, and results not shown). By contrast, all SMS sera that were positive by immunocytochemistry and/or western blotting were also positive by immunoprecipitation (results not shown). So anti-GAD antibodies in SMS patients are generally present at higher titres and recognize more distinct epitopes than anti-GAD antibodies in IDDM patients. However, in rare cases, anti-GAD antibodies in IDDM patients can recognize denatured GAD. Whereas the 7673 and S3 antisera recognize both the 65K form and the 64K α and β isoforms on western blots (Fig. 6*A*, lanes 2, 4, 5), human GAD autoantibodies as well as the monoclonal antibody GAD6 preferentially recognize the smaller 64K α and β isoforms (Fig. 2, lanes 7–11). The GAD6 antibody is specific for the smaller GAD isoform in brain¹⁶.

Discussion

The 64K autoantigen is the enzyme glutamic acid decarboxylase. We have demonstrated that the 64K autoantigen in IDDM is the enzyme GAD in pancreatic β cells. GAD, its product GABA, and the GABA-metabolizing enzyme GABA transaminase are present at high levels in pancreatic β cells as well as in GABA-secreting neurons¹⁹. GABA may act as an 'endocrine transmitter' in islets^{20–23} and this may explain why GAD is expressed in β cells. The β cell-specific expression of GAD in islets is consistent with its being an autoantigen associated with the selective destruction of pancreatic β cells in IDDM. GAD is a major autoantigen in SMS, a disease in which GABA-secreting neurons are thought to be affected^{10,11}. Thus pancreatic β cells and GABA-secreting neurons share a protein that is unusually susceptible to becoming an autoantigen, an observation that will

surely motivate studies not only into the pathogenesis of IDDM and SMS, but also into the mechanisms of generation of self-tolerance by the immune system, and its failures.

Differences in humoral autoimmunity to GAD in SMS and IDDM.

The humoral autoimmunity towards GAD in the two diseases in which GAD-expressing cells are either destroyed (IDDM) or thought to be affected (SMS), suggests a direct relationship between GAD autoantibodies and the diseases. Not all SMS patients with GAD antibodies develop IDDM, and only a small proportion of IDDM patients develop SMS, in spite of their having GAD autoantibodies for several years. This suggests that there are other components to both diseases. The development of SMS seems to involve a stronger antibody response to GAD and includes epitope recognition which is usually absent in IDDM, perhaps as a result of different characteristics of pancreatic β cells and GABA-secreting neurons. For example, the microenvironment of the CNS is protected by the blood-brain barrier and the antigenic requirements to initiate an autoimmune response across this barrier may be distinct. Pancreatic β cells express major histocompatibility complex (MHC) class I molecules²⁴ which are thought to present self-peptides to the immune system, whereas CNS neurons normally do not²⁵.

Subcellular localization of GAD in brain and islets and its role as an autoantigen. Synaptic-like vesicles have recently been identified in endocrine cells²⁶ and may be the storage sites of GABA in β cells, similar to synaptic vesicles in brain. Immunofluorescence studies have shown colocalization of GAD and membrane markers of these synaptic-like microvesicles (A.R., M.S. and P.D.C., unpublished results). We found that GAD in both brain and islets behave similarly with regard to hydrophobicity and compartmentalization, that is, both were detected in a soluble hydrophobic and a membrane-bound hydrophobic form. Although it cannot be excluded that GAD is expressed at the surface of β cells, it is more likely that the protein remains confined to the cytoplasmic space. Thus autoantibodies to GAD are unlikely to see the intact antigen on the surface of normal β cells. But peptides derived from the GAD molecule may be

expressed at the cell surface in the cleft of MHC class I antigens, and so be recognized by pathogenic T cells (ref. 27, and refs therein).

The identification of the 64K antigen as the enzyme GAD is of relevance in elucidating the role of this antigen in the development of IDDM. If the GAD autoantigen is shown to be critical

for the initiation of β -cell destruction, then an approach to therapy might be to develop ways of preventing or reversing the autoimmune response towards GAD in β cells of susceptible individuals. This would be similar to the successful prevention of autoimmune disease in experimental allergic encephalomyelitis²⁸. □

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Unusual radio arcs discovered in the radio source G318.9+0.4

J. B. Z. Whiteoak

School of Physics, The University of Sydney, NSW 2006, Australia

THE radio source G318.9+0.4 has been tentatively identified as a supernova remnant (SNR)^{1,2}. Here I report observations at 843 MHz revealing a remarkable network of arcs in the object which exclude it from any of the standard classes of SNRs. The arcs outline an approximately elliptical region enclosing a bright off-centre core component which has several curved extensions. Although the appearance of the arc structures is unprecedented, G318.9+0.4 might belong to a recently proposed class of non-thermal axisymmetric radio sources unrelated to SNRs³. Alternatively, the core component may indeed be a SNR, excited by the stellar remnant of the supernova event, with the arc emission arising through jet activity.

Supernova remnants (SNRs) are usually grouped into three classes. Plerions (~10 known objects) are remnants displaying a Crab-like or filled-centre morphology in which an embedded pulsar is thought to be the source of excitation. Shell-like remnants (150–200 objects) are characterized by an annular emission region, frequently only partially complete, believed to be the result of the supernova explosion as it expands into and interacts with the surrounding medium. Members of the so-called hybrid or composite class (~10 objects) combine both plerionic and shell-like features. The peculiar system of arc-like structures in the object G318.9+0.4 that I report here does not fit into any of the above classes.

The observations have been made as part of a survey of the southern galactic plane ($245^\circ \leq l \leq 360^\circ$ and $|b| \leq 1.5^\circ$) using the Molonglo Observatory Synthesis Telescope (MOST) in the radio continuum at 843 MHz^{4,5}. The resolution of the images shown

here is $43 \times 51 \text{ arcsec}^2$ (right ascension $\times$ declination) and the sensitivity is $\sim 0.5 \text{ mJy per beam}$. The good resolution and sensitivity of the MOST, combined with its low frequency and 1° field of view, produce a survey that is particularly useful in the study of SNRs.

One field in the MOST southern galactic plane survey contains the object G318.9+0.4, shown in Fig. 1a, b in greyscale and contour images. I have assumed that all the emission features are associated with a single entity, with the exception of the point-like objects which are probably extragalactic.

The 843-MHz flux density of G318.9+0.4 is $3.6 \pm 0.8 \text{ Jy}$ whereas the flux density from the 5-GHz survey of Haynes *et al.*⁶ is $3.0 \pm 0.3 \text{ Jy}^{1,2}$. This implies a spectral index of -0.1 ± 0.2 , typical of plerions and core-dominated hybrid SNRs. Although this spectral index alone does not prove conclusively that the emission has a non-thermal origin, confirmation is provided by Broadbent *et al.*² who conclude that the object is either extragalactic or a SNR because of the weakness of associated 60- μm infrared emission, and by Caswell and Haynes¹ who do not detect H recombination emission. H_2CO absorption measurements¹ provide a lower limit on the distance of 3 kpc, leading to a major-axis length of $>25 \text{ pc}$. Inspection of SERC-J plates and $\text{H}\alpha$ images^{7,8} reveals no optical counterpart.

Although an extragalactic origin cannot be ruled out, G318.9+0.4 seems to be galactic, both on account of its large angular size ($30 \times 14 \text{ arcmin}^2$) and its low galactic latitude. Using 5-GHz observations at a resolution of 4 arcmin, Caswell and Haynes¹ suggested that G318.9+0.4 might be a SNR resembling the Crab nebula because of its apparent plerionic morphology. But the detection of arc structures in addition to a core component in the MOST image implies that if G318.9+0.4 is a SNR, it might instead be classified as a member of the hybrid class.

Figure 1a, b shows that the core of G318.9+0.4 is composed of a bright patch of diameter $\sim 5 \text{ arcmin}$, giving it a size of $>4 \text{ pc}$, with unusual faint curved extensions from its southern, western and eastern sides. The contour map shows that the patch

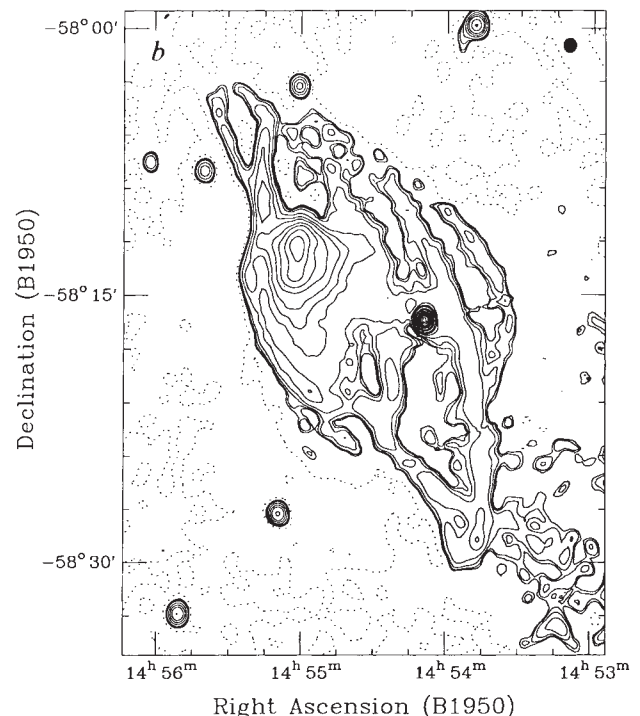
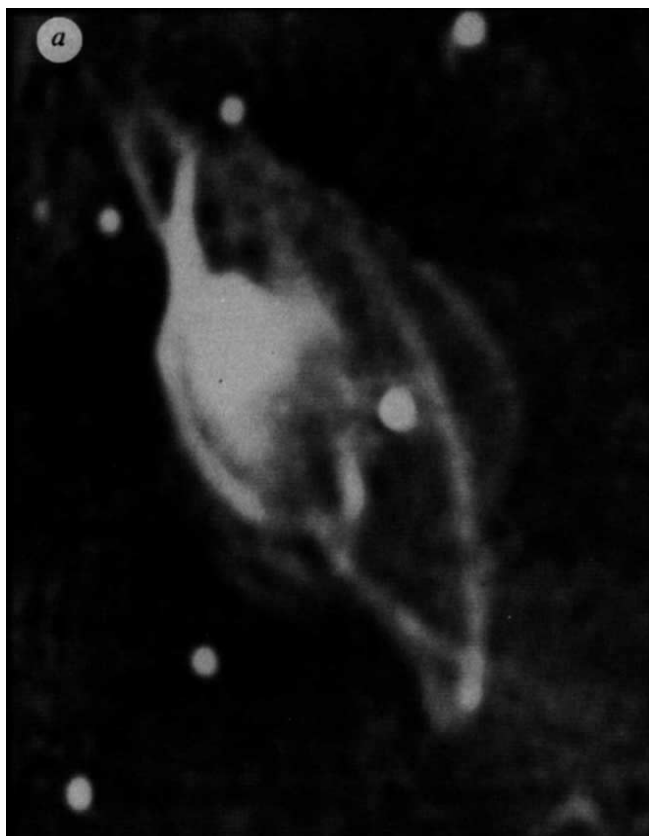


FIG. 1 a, An image of G318.9+0.4 made with the MOST at 843 MHz. North is to the top and east to the left; b, the corresponding contour diagram, with levels drawn at -1 (dashed), 2.5, 3, 4, 6, 12, 20, 30, 50, 70, 100, 130 mJy per beam. Maximum and minimum flux densities are 157.1 and $-9.6 \text{ mJy per beam}$, respectively.

contains a ridge extended in the north-south direction with an emission maximum at its northern end. The eastern extension from the patch is seen to extend into, but not beyond, the arc structure just to its east, and to project back to the core maximum.

Around the core component is a system of unusual arc structures. None of the arcs is concave away from the core, and most are noticeably concave towards it. The brightness distribution along each arc is not uniform, and Fig. 1*a, b* shows that the arc lying closest to the core (to its east) has the highest surface brightness of all the arcs. Several of the arcs have very low brightness, and there are small gaps or endpoints in the emission which may reflect insufficient sensitivity to reveal the complete structure. Where the arcs overlap, there is often a local brightness maximum, indicating that the arcs are optically thin at 843 MHz. Lying adjacent to the outer side of the arc envelope in the southeast is a region of weak diffuse emission, possibly a continuation of the southern core extension.

The complexity of the arcs of G318.9+0.4 challenges existing SNR models. The arcs are quite atypical of shell-like SNRs, making it difficult to assign G318.9+0.4 to the hybrid class of remnants despite its plerionic component. In the basic model of a shell-like SNR the observed emission arises from a thin spherically symmetric shell representing the expansion of the supernova shock into the pre-existing medium and any mass lost by the progenitor star. The commonly observed arc structures in SNRs would then originate from the parts of the shell viewed edge-on. In conjunction with the deforming tendencies of an inhomogeneous medium, this model can account for the observed brightness distributions of a large number of shell SNRs. The number of arcs of G318.9+0.4 and their configuration are not consistent with such a scheme, however, and it seems unlikely that their unusual character could be due to a particular geometry of the surrounding medium.

Other discrepancies with the shell model include both the small lateral extent of the arcs and the high elongation of the object. Most known shell SNRs have substructure in their shells, and typically a ratio of shell thickness to radius of curvature of 0.3⁹. But the arcs of G318.9+0.4 are unresolved in thickness (thickness ≤ 30 arcsec), show no structure and have a thickness ratio of <0.05 (for an effective radius of 10 arcmin). The elongation of the object is unusual as the axial ratio (minor/major) of a shell SNR is typically 0.79¹⁰, whereas that of G318.9+0.4 is 0.43.

A comparison of the gross properties of G318.9+0.4 with those of known SNRs also indicates peculiar behaviour. The mean surface brightness interpolated to 1 GHz is $(1.7 \pm 0.4) \times 10^{-21} \text{ W m}^{-2} \text{ Hz}^{-1} \text{ sr}^{-1}$, lower than that of any hybrid in the remnant list of Green¹¹. With an 843-MHz flux density of $(1.8 \pm 0.2) \text{ Jy}$, however, the core component has a mean 1-GHz surface brightness of $(9.6 \pm 1.0) \times 10^{-21} \text{ W m}^{-2} \text{ Hz}^{-1} \text{ sr}^{-1}$, which is typical of pure plerions¹¹. These measurements further cast into doubt the interpretation of the arcs as part of a normal SNR, and perhaps support the possibility that the core emission is a pulsar-driven component. In this respect, G318.9+0.4 resembles

CTB80¹², a peculiar SNR candidate possessing a plerionic component excited by a pulsar^{13,14} that is also associated with several unusual arc-like structures.

Some models¹⁵ propose that the commonly observed SNR 'shell' brightness distributions can be accounted for not by a spherical blast wave, but by relativistic beams from a rotating neutron star that trace out annular regions on the sky as the star rotates. Such a mechanism could be responsible for the arcs of G318.9+0.4 because the arcs may represent parts of thin annuli lying in planes almost parallel to our line of sight. The high elongation, cusped ends and the tendency for the arcs to form opposing pairs of similar curvature are consistent with such a picture. No complete annulus is evident, however, and it is difficult to reconcile all the arcs with this model.

Another possibility is that G318.9+0.4 might not be a SNR at all, but rather another member of the class of non-thermal axisymmetric objects proposed by Becker and Helfand^{3,16}. G318.9+0.4 displays some similarities to the prototype, G357.7-0.1, namely several partial ring-like structures and a possible compact component represented by the point-source lying in the arc envelope. Becker and Helfand³ note that the arcs of G357.7-0.1 resemble concentric 'smoke-rings' of different diameters, and suggest that this may be the result of intermittent bursts from the compact object as it travels along the axis. But the similarity of G318.9+0.4 to the members of the axisymmetric class is not complete: its arc emission is not as diffuse as that of the axisymmetric objects, its axial symmetry is less prominent, and there is little evidence of the characteristic surface-brightness gradient expected along the axis.

The presence of a plerionic component in G318.9+0.4 is compatible with the existence of a neutron star or other compact object, and the approximately elliptical arc envelope, the narrow arc thickness, and the off-centre location of the core might then be explained by precessing jets from such an object coupled with the effects of non-uniformities in the local medium. According to this picture, the character of the arcs of G318.9+0.4 might be explained by a theory similar to that accounting for the 'corkscrew' behaviour of the ejecta from SS433¹⁷, although the precession axis of G318.9+0.4 may lie closer to our line of sight than that of SS433. The extensions in the core component might then be manifestations of the beamed emission. An interaction between the core and arcs is suggested by the enhanced emission of the eastern arc, the arc lying closest to the core. A search for pulsed emission carried out using the MOST was unsuccessful.

Further studies of the object are clearly needed. H I measurements should be carried out to determine whether the object could be an external galaxy. If the object does lie in the Galaxy and if the core is excited by a neutron star, X-ray observations might reveal emission coincident with the object. Radio-frequency polarization measurements would confirm the non-thermal character and reveal the direction of the magnetic field in the core and arc components, and measurements of the radio spectrum at various points in the source might provide further clues to the nature of this unique object. $\square$

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Ubiquitous interstellar diamond and SiC in primitive chondrites: abundances reflect metamorphism

Gary R. Huss

Enrico Fermi Institute, University of Chicago, Chicago, Illinois 60637-1433, USA

THE nature and distribution of interstellar grains in meteorites reflect both the material inherited from the Sun's parent molecular cloud and processes that operated in the early Solar System. The discovery of interstellar diamond and silicon carbide (SiC) in carbonaceous chondrites^{1,2} demonstrated that presolar grains were incorporated into at least some meteorites. Here I show that interstellar diamond and SiC were incorporated into all chondrite groups. Abundances rapidly go to zero with increasing metamorphic grade (with diamond typically surviving better than SiC), suggesting that metamorphic destruction is responsible for the apparent absence of these grains in most chondrites. In unmetamorphosed chondrites, abundances normalized to matrix content are similar for different classes (500–1,000 p.p.m. for diamond; 10–18 p.p.m. for SiC). Diamond samples from chondrites of different classes have remarkably similar noble-gas contents and isotopic compositions, although constituent diamonds may have come from many sources. SiC seems to be more diverse, in part because grains are large enough to measure individually, but average characteristics seem to be similar from meteorite to meteorite. These observations suggest that various classes of chondritic meteorites sampled the same Solar-System-wide reservoir of interstellar grains.

To assess the distribution of interstellar diamond and SiC as a function of meteorite class and metamorphic history, I studied 10 primitive ordinary (LL, L, H), 4 carbonaceous (CI, CV) and 3 enstatite (E) chondrites. Meteorite samples were processed using variants of the procedure in ref. 2 to yield first oxidized HF-HCl residues, and then diamond and SiC-spinel separates, which were measured for noble gases by stepped pyrolysis and

examined by scanning electron microscopy and energy dispersive X-ray analysis.

Diamond separates from all chondrite groups have the same physical appearance and behaviour during chemical processing. In most primitive chondrites (those that have suffered little alteration), diamond separates consist of a single interstellar component, C δ (ref. 1). C δ is the primary (only?) carrier of exotic Xe-HL, which is enriched twofold in the lightest and heaviest isotopes compared to normal 'planetary' Xe (refs 1, 2). Not only are the noble gases distinctive, but C δ separates have remarkably similar noble-gas contents and isotopic compositions across the meteorite classes. This similarity, illustrated for Xe in Table 1, extends to isotope variations observed during stepped pyrolysis³ and suggests that the C δ in ordinary, enstatite and carbonaceous chondrites came from a single Solar-System-wide reservoir. Diamond separates in the most primitive meteorites (CI, CM, LL3.0) contain another noble-gas component of normal isotopic composition that is released at 300–900 °C, compared to 1,000–1,600 °C for C δ . Its carrier, designated C ζ (ref. 4), resembles C δ in colour, grain size and chemical (but not thermal) resistance, and may consist of diamond-like hydrocarbon. Here I focus on C δ , which can be traced by its unusual noble gases.

To minimize errors owing to sample loss, it is desirable to estimate C δ abundances as early as possible in the chemical processing. Because of the constancy shown in Table 1, accurate estimates of C δ abundance can be obtained from oxidized HF-HCl residues. Oxidation removes the 'planetary' noble gases (>90% of the Ar, Kr and Xe), revealing the distinctive noble gases of C δ (and SiC). Xe isotopic composition (especially ¹³⁶Xe/¹³²Xe) is the most sensitive indicator of C δ , and abundances are calculated as follows

$$C\delta(\text{p.p.m.}) = \frac{\left(\frac{^{136}\text{Xe}}{^{132}\text{Xe}}\right)_{\text{meas.}} - \left(\frac{^{136}\text{Xe}}{^{132}\text{Xe}}\right)_{\text{plan.}}}{\left(\frac{^{136}\text{Xe}}{^{132}\text{Xe}}\right)_{C\delta} - \left(\frac{^{136}\text{Xe}}{^{132}\text{Xe}}\right)_{\text{plan.}}} \frac{[^{132}\text{Xe}]_{\text{meas.}}}{[^{132}\text{Xe}]_{C\delta}} \times F \times 10^6$$

where F is the mass fraction of the meteorite made up by the residue. The measured ratio and concentration come from the oxidized residue, the ratio and concentration for C δ are the mean values from Table 1, and the 'planetary' ratio (0.310–0.326) comes from Xe in unoxidized samples of the same meteorite or another meteorite of the same class. The calculation assumes that C δ is the only carrier of Xe-HL in meteorites and that diamonds have not lost their noble gases. I typically recovered 70–90% of the C δ calculated to be present in the oxidized residues and did not find any new Xe-HL carrier in complementary fractions, so the first assumption seems valid. Although C δ abundance changes with metamorphic grade (see below), the Ne/Xe ratio remains nearly constant, indicating that C δ is destroyed rather than degassed in meteorites.

Estimates of C δ abundance for meteorites in this study are combined with estimates based on literature data from oxidized residues in Table 2. I have taken special care to reduce both overall sample losses and preferential diamond loss during chemical processing. Where cross-checks are available, my values of C δ abundances tend to be more than a factor of two higher than those based on literature data. Uncertainties associated with sample preparation are not included in the errors in Table 2, but entries subject to excessive losses have been indicated. Table 2 shows that interstellar diamonds are present in primitive members of all of the main chondrite classes.

Interstellar SiC was first found in CM2 chondrites^{2,5}, but it has now been identified^{6–9} in ordinary, E, CI and CV3 chondrites as well (CO3 chondrite studies are in progress). Interstellar SiC is the principal carrier of Ne-E(H) (Ne-E released at high temperatures during stepped pyrolysis), and Kr-S and Xe-S (Kr and Xe produced by the astrophysical s-process)^{2,10}. Ne-E(H), with its extreme ²⁰Ne/²²Ne (assumed to be zero), is the most

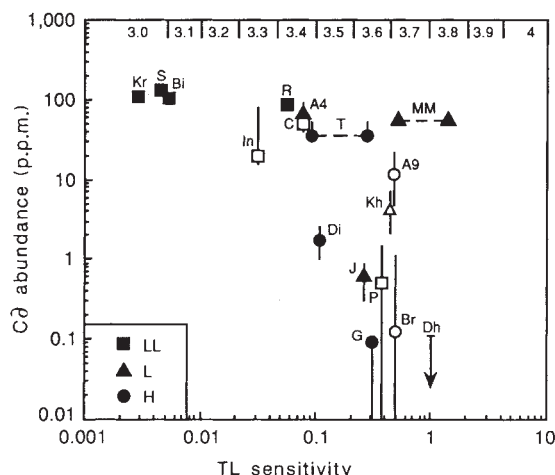


FIG. 1 C δ abundances for type 3 ordinary chondrites are plotted against thermoluminescence (TL) sensitivity, a measure of metamorphic grade^{26,27}. Open symbols are literature-based abundance estimates and plotted errors are larger than those in Table 2 owing to systematic uncertainties described in text. The top scale correlates petrologic type with TL sensitivity. The range in thermoluminescence for Tieschitz may be due to an unusual matrix component²⁸, but no explanation is known for Mezö-Madaras. Kr, Krymka; S, Semarkona; Bi, Bishunpur; In, Inman; R, Ragland; C, Chainpur; A4, ALHA77214, T, Tieschitz; Di, Dimmitt; A9, ALHA77299, MM, Mezö-Madaras; Kh, Khohar; J, Julesburg; P, Parnallee; G, Grady; B, Brownfield; Dh, Dhajala.

TABLE 1 Xe contents and isotopic compositions of diamond separates from primitive chondrites

Meteorite class	^{132}Xe $10^{-8} \text{ cm}^3 \text{ g}^{-1}$	$\frac{^{124}\text{Xe}}{^{132}\text{Xe}}$	$\frac{^{126}\text{Xe}}{^{132}\text{Xe}}$	$\frac{^{128}\text{Xe}}{^{132}\text{Xe}}$	$\frac{^{129}\text{Xe}^*}{^{132}\text{Xe}}$	$\frac{^{130}\text{Xe}}{^{132}\text{Xe}}$	$\frac{^{131}\text{Xe}}{^{132}\text{Xe}}$	$\frac{^{134}\text{Xe}}{^{132}\text{Xe}}$	$\frac{^{136}\text{Xe}}{^{132}\text{Xe}}$
Orgueil	CI								
C ζ †		32.4	0.467 (2)	0.410 (2)	8.10 (2)	106.1 (3)	15.93 (5)	82.4 (2)	38.5 (1)
C δ †		17.4	0.733 (4)	0.529 (3)	8.80 (3)	105.0 (3)	15.62 (5)	83.7 (3)	56.5 (2)
Semarkona	LL3.0								
C ζ †		13.0	0.476 (4)	0.413 (4)	8.15 (2)	115.4 (3)	15.86 (5)	82.5 (2)	39.9 (1)
C δ †		20.1	0.732 (4)	0.522 (3)	8.83 (3)	105.2 (3)	15.63 (5)	83.8 (3)	56.4 (2)
Ragland	LL3.4	25.3	0.773 (4)	0.546 (3)	9.01 (3)	109.7 (3)	15.58 (5)	84.0 (3)	59.4 (2)
ALHA 77214	L3.4	24.5	0.775 (4)	0.540 (4)	8.95 (3)	107.3 (3)	15.61 (5)	83.9 (3)	59.0 (1)
Dimmitt (1800 C)	H3.5	25.5	0.767 (13)	0.539 (15)	8.99 (8)	108.4 (4)	15.64 (8)	83.4 (3)	58.1 (5)
Qingzhen‡	E3	24.0	0.772 (5)	0.553 (4)	8.99 (3)	106.4 (3)	15.72 (5)	83.7 (3)	58.8 (1)
Indarch‡	E3-4	30.9	0.770 (4)	0.545 (3)	9.10 (3)	106.7 (3)	15.79 (5)	83.7 (3)	58.7 (2)
Allende	CV3	25.1	0.790 (5)	0.551 (5)	9.11 (3)	107.3 (3)	15.58 (5)	84.3 (3)	59.9 (2)
Vigarano	CV3	20.5	0.770 (4)	0.540 (3)	8.99 (3)	111.9 (3)	15.59 (5)	84.0 (3)	58.7 (2)
Mean§		25 (3)	0.774 (8)	0.545 (6)	9.02 (6)	105 (1)	15.60 (3)	83.9 (3)	58.9 (6)

Isotopic ratios have been multiplied by 100. ^{132}Xe concentrations are subject to 10% systematic uncertainty. Numbers in brackets are the 1σ uncertainties.

* Includes contribution from radiogenic ^{129}Xe acquired from the host meteorite.

† Data for C ζ and C δ are measured compositions of the low- and high-temperature gas releases, respectively. Gas concentrations are low owing to the presence of the complementary carrier in the measured diamond separate. Isotope ratios reflect incomplete separation of two components.

‡ Samples contaminated by a small amount of SiC, which raises $^{128}\text{Xe}/^{132}\text{Xe}$ and $^{130}\text{Xe}/^{132}\text{Xe}$ and lowers $^{124}\text{Xe}/^{132}\text{Xe}$, $^{126}\text{Xe}/^{132}\text{Xe}$, $^{134}\text{Xe}/^{132}\text{Xe}$ and $^{136}\text{Xe}/^{132}\text{Xe}$ through addition of Xe-S.

§ Excluding Orgueil and Semarkona because of contributions from C ζ and excluding Qingzhen and Indarch from the $^{130}\text{Xe}/^{132}\text{Xe}$ average. $^{129}\text{Xe}/^{132}\text{Xe}$, which is affected by radiogenic ^{129}Xe from the meteorite, is the lowest observed value among temperature fractions.

TABLE 2 Abundances of interstellar C δ diamonds and SiC in primitive chondrites

Meteorite	Class and type*	Diamonds in bulk meteorite (p.p.m.)	SiC in bulk meteorite (p.p.m.)	$^{22}\text{Ne-E(H)}$ $10^{-12} \text{ cm}^3 \text{ g}^{-1}$	Diamonds in matrix (p.p.m.)	SiC in matrix (p.p.m.)
Orgueil	CI	941 (94)	14.1 (1.5)	2,250 (234)	941	14.1
Semarkona	LL3.0	131 (13)	1.52 (16)	243 (26)	838	9.7
Krymka	LL3.0	109 (11)	0.39 (6)	62 (10)	801	2.9
Bishunpur	LL3.1	102 (10)	1.36 (14)	217 (23)	734	9.8
Inman ^{19,20}	LL3.3	20 (3)†	0.33 (9)	52 (16)	150†	2.4
Ragland	LL3.4	86 (9)	0.093 (10)	15 (2)	637	0.7
Chainpur ²¹	LL3.4	50 (5)†	0.64 (32)†	103 (50)†	310†	4†
Parnallee ²¹	LL3.6	0.5 (3)†	<0.0015†	<0.24†	3.6†	—
ALHA 77214	L3.4	64 (10)†	0.008 (1)†	1.3 (2)†	464†	0.06†
Mezö-Madaras	L3.7	54 (5)	0.081 (16)	12.9 (2.6)	545	0.8
Julesburg	L3.6	0.6 (3)	0.00056 (36)	0.090 (55)	4.4	0.04
Khojar ¹⁹	L3.6	4 (2)†	<0.04†	<6†	29†	—
Tieschitz	H/L3.4-6	36 (4)†	0.063 (14)	10 (2)	240†	0.42
Dimmitt	H3.5	1.8 (8)	0.0010 (3)	0.16 (5)	13	0.007
Grady	H3.6	0.08 (6)	<0.006	<1	0.6	—
Brownfield ²²	H3.6	0.13 (3)†	—	—	1†	—
ALHA 77299 ¹⁹	H3.7	12 (2)†	<0.11†	<17†	87†	—
Dhajala ¹⁹	H3.8	<0.1†	<0.044†	<7†	—	—
Qingzhen	E3	67 (7)	1.6 (2)	254 (26)	478	11
Indarch	E3-4	50 (10)	1.3 (2)	207 (21)	500	13
Abee	E4-5	<0.3	<0.006	<1	—	—
Murray ²	CM2	400 (40)†	4.2†	670†	800†	8.4†
Murchison ²³	CM2	>360†	9.0†‡	1,440†‡	>720†	18†
Kainsaz ²⁴	CO3.1	165 (5)†	—	—	550†	—
Lancé ²⁴	CO3.4	53 (5)†	—	—	162†	—
Ornans ²⁴	CO3.3	17 (2)†	—	—	42†	—
Grosnaja ²⁵	CV3	440 (50)†	—	—	858†	—
Leoville	CV3	491 (49)	>0.18	>28	1,400	>0.51
Vigarano	CV3	497 (50)	>0.07	>11	1,440	>0.20
Allende	CV3	>250†	—	—	>650†	—

Uncertainties on abundances do not include unknown and potentially large (for literature data) sample losses during chemical processing.

* Petrologic types for ordinary chondrites from refs. 26, 27 except for Julesburg, Dimmitt and Grady, which were provided for our samples by David Batchelor (Univ. Arkansas). Petrologic types for CO chondrites from ref. 28.

† Results of samples subject to excessive losses.

‡ Data for Ne-E(H) in Murchison provided by S. Amari.

sensitive tracer of SiC and can be resolved from Ne in C δ (Ne-A2, ref. 2) and spallation Ne, the other Ne components in properly oxidized residues, to give Ne-E(H) abundances in a manner analogous to that previously described for C δ Xe. Converting Ne-E(H) content to SiC abundance is less certain, because the Ne-E(H) content in SiC varies with grain size by a factor of about seven around a mean value of $\sim 16,000 \times 10^{-8} \text{ cm}^3 \text{ g}^{-1}$ (S. Amari, R. S. Lewis and E. Anders, unpublished data) and because some laboratory treatments seem to remove part of the Ne-E(H). Because meteorites contain similar mixes of grain sizes and the suspect treatments were not used on my samples, these uncertainties should affect abundance estimates by less than a factor of two. A potentially more serious problem for abundance determinations is degassing of SiC, either before incorporation into the meteorite or during subsequent heating. At present I cannot address this uncertainty, but work is under way to improve the abundance determinations.

Table 2 shows Ne-E(H) contents and SiC abundances for meteorites that I have analysed as well as those for others based on literature data. For Vigarano and Leoville, the oxidized residues of which also contain appreciable 'planetary' Ne, Ne-E(H) contents could not be accurately determined, but lower limits were established from high-temperature steps. Errors in Table 2 do not include the uncertainty in the Ne-E content of SiC. As Table 2 shows, interstellar SiC was apparently as widely distributed as C δ diamond in the early Solar System.

The abundances of C δ and SiC compiled in Table 2 vary by factors of $>10^3$. This variation correlates with metamorphic grade and with the percentage of fine-grained 'matrix'¹¹, the principal site of volatile constituents of chondrites and probable site of interstellar grains. Within a meteorite class, abundances correlate with petrologic type, a scale of metamorphic grade. In ordinary chondrites, C δ abundances decline slowly from ~ 130 to ~ 50 p.p.m. in petrologic types 3.0–3.4, but drop sharply to <2 p.p.m. in types 3.5–3.6; no indication of C δ has been found in types ≥ 3.8 (Fig. 1). The steep drop in C δ abundance correlates with a change in matrix appearance from fine-grained and opaque in types 3.0–3.4 to largely recrystallized in types 3.5–3.6¹¹, suggesting that matrix recrystallization destroys

diamond. SiC abundances in ordinary chondrites fall more rapidly across types 3.0–3.4 than do those of C δ (Fig. 2) and are less well correlated with petrologic type (Table 2). Like C δ , SiC has vanished in types ≥ 3.8 . Apparently SiC is more susceptible to metamorphic destruction in ordinary chondrites in spite of its larger grain size (<0.1 – $10 \mu\text{m}$ compared to $\sim 25 \text{ \AA}$ for diamond), perhaps through reaction with surrounding oxides. Similarly, abundances decrease with increasing petrologic type in E and CO3 chondrites (Table 2), but in the highly reduced E chondrites SiC survives better than in ordinary chondrites.

Between meteorite classes, most of the abundance variation reflects differences in matrix content. Matrix-normalized abundances for C δ and SiC are shown in Table 2 and, for the meteorites in this study and two CM2 chondrites, are plotted against one another in Fig. 2. Matrix abundances used in the normalization came from petrographic observations (refs. 11–13, and my unpublished data), except those for CM2 chondrites where aqueous alteration has obscured the amount of original matrix¹⁴ and 50% matrix (based on a chemical definition¹⁵) was used. Although differences in the definition and/or method used to obtain matrix abundance may result in uncertainties in absolute matrix-normalized C δ and SiC abundances of perhaps 25%, relative uncertainties within a meteorite group are probably $<10\%$. Matrix-normalized C δ and SiC abundances for the least altered ordinary and E chondrites, and for CM2 chondrites, are similar to those in Orgueil (CI), which consists entirely of 'matrix' (Fig. 2). CI chondrites have essentially the same non-volatile element composition as does the Sun¹⁶, suggesting that they representatively sampled the material from which the Sun (and Solar System) formed. The similarity of matrix-normalized diamond and SiC abundances in the least altered members of several meteorite classes to those in Orgueil suggests that these primitive meteorites sampled the same reservoir of interstellar material, probably the mixture inherited from the Sun's parent molecular cloud.

Residual variation in matrix-normalized C δ and SiC abundances in unmetamorphosed chondrites may reflect pre-accretionary processing. For example, CV3 chondrites, the matrices of which have more refractory mineralogies and chemical compositions than those in ordinary, CM2 and CI chondrites (see, for example, refs 11, 17, 18), have higher matrix-normalized diamond abundances than does Orgueil (Fig. 2). If dust that was to become the matrices of CV3 chondrites became more refractory through evaporation of volatile constituents before accretion and diamonds acted as inert refractory constituents in the dust, then one would expect to find enhanced C δ abundances in CV3 matrices¹⁸. Lower C δ abundances in E chondrites and apparently lower SiC abundances in CV3 chondrites (SiC abundances in CV3s are not well determined but Ne-E(H) concentrations are unlikely to have been underestimated by a factor of 50) may also reflect pre-accretionary processing.

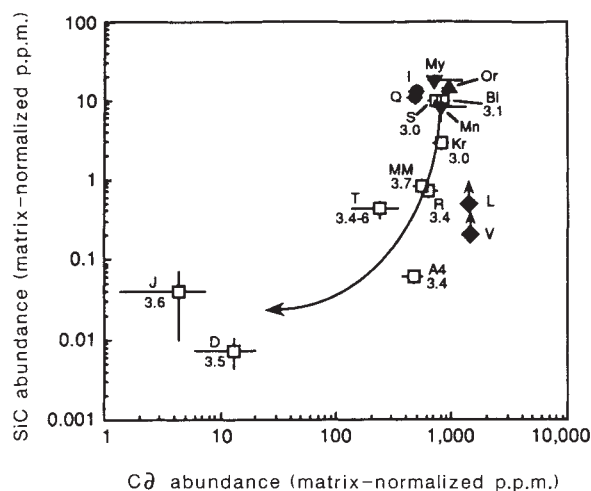


FIG. 2 The most reliable matrix-normalized C δ and SiC abundances from Table 2 are plotted. Orgueil (▲), thought to be a chemically representative sample of Solar System material, plots in the upper right, along with matrices of other highly primitive meteorites: CM2 chondrites (▼), E3 chondrites (●), and the least metamorphosed ordinary chondrites (□). Petrologic types for ordinary chondrites are shown, as is the trend generated by ordinary-chondrite metamorphism, with SiC lost first, then C δ . CV3 chondrites (◆) have higher C δ abundances than other primitive meteorites and, probably, lower SiC abundances (shown as lower limits). I, Indarch, Q, Qingzhen, My, Murray, Mn, Murchison, Or, Orgueil, L, Leoville, V, Vigarano. Others the same as in Fig. 1.

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Backscattering from frost on icy satellites in the outer Solar System

Anne Verbiscer, Paul Helfenstein & Joseph Veverka

Center for Radiophysics and Space Research, Cornell University, Ithaca, New York 14853, USA

THE surfaces of most satellites in the outer Solar System are covered, at least in part, by water frost¹. Extensive analysis of the photometry of these icy satellites has shown that their surfaces are remarkably backscattering (refs 2–5; A.V. and J.V., manuscript in preparation). We present two extreme models of how frost and ice might be intermixed on a typical satellite surface: areal (checkerboard mixing) and intimate mixing. Applying such models to selected representative satellite data, we find that the frost component of the surfaces of these outer satellites must itself be backscattering, unlike its terrestrial counterpart. The difference may arise because frost particles can have much more complex internal textures under the low-temperature and low-gravity conditions of the outer satellites than is the case on Earth.

The surfaces of most satellites in the outer Solar System are covered at least in part by water frost¹. This is the case for Europa, Ganymede and Callisto in the Jupiter system, for most of the satellites of Saturn, with the exception of Titan and probably Phoebe, and it is also true for the five larger satellites of Uranus. In one case, that of Saturn's Enceladus, the surface layer of water frost must be almost pure because the very high albedo precludes the existence of dark material in the layer into which light penetrates⁶. In general, however, the frost is mixed with darker, more opaque, components, the composition of which remains to be characterized but which are widely believed to be similar, at least in terms of optical properties, to the surfaces of carbonaceous asteroids⁷. (This generalization may not apply to Europa, where at least some of the darker component may be sulphur ultimately derived from Io⁸.) The darker component is responsible for lowering absolute albedos and for decreasing the prominence of the water-ice bands at 1.5 and 2 μm .

When the Hapke model⁹ is used to describe photometry of outer satellites one always finds that the observations imply that the average regolith particle is backscattering, rather than forward scattering as is terrestrial water frost. The Henyey–Greenstein parameter g , defined as $\langle \cos \theta \rangle$ where θ is the scattering angle, describes the asymmetry of the scattering phase function of an average regolith particle. For isotropic scattering g is zero. Positive values correspond to forward scattering and negative values to backscattering. The satellites listed in Table 1 have values of g between -0.1 and -0.5 , and are therefore slightly to moderately backscattering. Also shown are values of g determined by fitting Hapke's model to observations of terrestrial snow and values calculated from Mie theory for large ice spheres using the optical constants of water ice¹⁰. Although it

may be argued that Mie calculations are not relevant to the complex particle shapes known to occur in natural frost layers, the important point is that the snow and frost layers for which we have measurements do have highly positive asymmetry parameters (forward scattering).

Although in some cases phase-angle coverage of observations may not be sufficient to exclude completely the possibility of any forward scattering from icy satellite surfaces, the fact remains that these surfaces have strong backscattering lobes which their terrestrial counterparts lack. If a two-term Henyey–Greenstein fit is applied to available data from Rhea (a satellite of Saturn), for which phase angles range from 2° to 135° , the partition coefficient determined by the fit weights the asymmetry parameters such that the forward-scattering component (+0.31) only contributes 20% of the solution. The backscattering g (-0.33) dominates the solution and is similar to the one-term value (-0.29). Also, if we reduce the phase-angle coverage of a surface known to be forward scattering (in Middleton and Mungall's¹¹ data for fresh snow, for example) to the same range of phase angle as the Rhea data ($<135^\circ$), and refit the data using the Hapke equation, the asymmetry parameter is still positive.

The backscattering behaviour of a typical icy satellite cannot be explained by a model in which the surface (at scales below the resolution limit of the observations) is a two-component 'checkerboard', one component of which is 'normal' frost or snow (Middleton and Mungall's¹¹ 'fresh snow' analysed in terms of Hapke's model¹²) and the other 'typical' dark material (characterized by Hapke parameters derived from observations of asteroid Ceres¹³). Whole-disk phase curves for such mixtures cannot be made to match observational data from icy satellites. To change the forward scattering behaviour of the frost to the backscattering behaviour of the satellite as a whole requires unacceptably (in terms of albedo) large fractions of dark material.

Nor can the backscattering behaviour be explained by a model in which frost and dark material particles are mixed intimately, unless the frost component itself is backscattering. For a two-component intimate mixture of particles, the following equations give the resulting single-scattering albedo

$$\tilde{\omega}_0 = \frac{\alpha_1 Q_{s1} N_1 + \sigma_2 Q_{s2} N_2}{\sigma_1 Q_{e1} N_1 + \sigma_2 Q_{e2} N_2} \quad (1)$$

and the Henyey–Greenstein asymmetry parameter

$$\tilde{g} = \frac{\sigma_1 Q_{s1} N_1 g_1 + \sigma_2 Q_{s2} N_2 g_2}{\sigma_1 Q_{s1} N_1 + \sigma_2 Q_{s2} N_2} \quad (2)$$

where σ is the average geometric cross-section of a particle, Q_e the particle extinction efficiency, Q_s the particle scattering efficiency, and N the number of particles per unit volume. Equations (1) and (2) can be combined and the dependencies on σ , Q_e , Q_s and N eliminated to derive an expression for the asymmetry parameter $\tilde{g}$ of the mixture as a function of the g_n and $\tilde{\omega}_{0n}$ of the endmembers as well as the single-scattering albedo $\tilde{\omega}_0$ of the mixture

$$\tilde{g} = \frac{\tilde{\omega}_{01} g_1 (\tilde{\omega}_0 - \tilde{\omega}_{02}) + \tilde{\omega}_{02} g_2 (\tilde{\omega}_{01} - \tilde{\omega}_0)}{\tilde{\omega}_0 (\tilde{\omega}_{01} - \tilde{\omega}_{02})} \quad (3)$$

In Fig. 1 we have plotted a family of $\tilde{\omega}_0$ – g relationships given by equation (3) for mixtures in which the 'dark' component is 'Ceres material' ($\tilde{\omega}_0 = 0.06$, $g = -0.4$) and the bright component ranges from the 'normal' frost discussed above ($\tilde{\omega}_0 = 0.999$, $g = +0.52$) to hypothetical frosts that are gradually more backscattering (more negative g). This family of curves is indicated by solid lines. Also shown is a second family of curves (dashed lines) in which we have chosen the dark component to be even more backscattering ($g = -0.6$ instead of -0.4) to illustrate that the precise value of g for the dark component has little effect on the discussion below. We have also included data points for

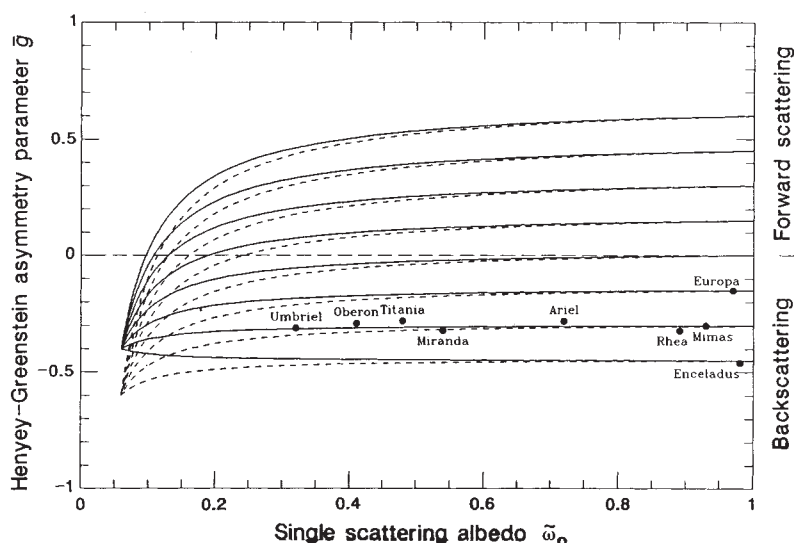


FIG. 1 Single-scattering albedos ($\tilde{\omega}_0$) and Henyey-Greenstein asymmetry parameters (g) for intimate mixtures of two dark endmembers ($\tilde{\omega}_0 = 0.06$, $g = -0.4$ solid lines; $g = -0.6$ dashed lines) and several bright endmembers ($\tilde{\omega}_0 = 0.999$, $g = -0.45$ to $+0.6$). Values of g for the bright 'frost' endmember can be read from intercepts on the right vertical axis. Observed values of $\tilde{\omega}_0$ and g for a number of icy satellites (solid circles) lie near the $g = -0.3$ line, indicating the icy component of their regoliths is strongly backscattering.

various icy satellites taken from Table 1. It is clear that the observations cannot be matched unless the frost component is assumed to be backscattering. Although we do not claim that all satellite surfaces are mixtures of the same two endmember materials, present in different proportions, it does seem that all of the data can be fitted approximately if the frost component has a g value of about -0.3 . The actual value of g is a function of the characteristics assumed for the endmember dark material, which of course remain uncertain. Thus we are led to the remarkable conclusion that the water frost on outer satellites is dominantly backscattering, unlike its terrestrial analogues.

Frost particles are forward scattering at visible wavelengths because they are very transparent¹⁴. Backscattering behaviour can be increased by increasing absorption, but as this also decreases $\tilde{\omega}_0$ and the optical path length, such a solution is unacceptable in cases of such high-albedo objects as Enceladus and Europa¹⁷. Backscattering particles have also been produced by filling them with internal scatterers¹⁵. Thus, a likely alternative is that the backscattering behaviour in icy regoliths is caused by the fact that the regolith particles occur in aggregates of very complex texture so that the backscattering is caused by the same combination of effects that cause what are loosely called 'opposition effects'¹⁶. It is known that very intricate textures of ice grains can be produced under vacuum and low-temperature conditions, and as long as temperatures remain low, preventing the annealing of sharp boundaries, such textures can be

preserved^{1,18}. It should therefore be possible to produce and maintain backscattering frosts under suitable conditions in the laboratory. At the higher temperatures representative of natural conditions in the Earth's cold regions, such textures should be destroyed by migration and redeposition of water vapour within the frost layer¹⁹.

But conditions at the surfaces of icy satellites are much different than those on Earth. Absolute temperatures are far below the freezing point of water, and atmospheric pressures are generally negligible. Under these conditions, many physical mechanisms exist for the origin and perpetuation of intricate particle textures²⁰. For example, fluid volcanism involving water and in some cases other volatiles such as ammonia and methane has been widely proposed to account for extensive icy resurfacing of Ganymede, Europa, Enceladus, Ariel and other icy satellites. Fluid volcanism in a vacuum should be accompanied by violent boiling, frothing, flash vaporization and freezing of the effusive liquid²¹. Laboratory experiments have demonstrated that rapid freezing owing to the removal of latent heat from water magmas during flash vaporization can produce complex, highly vesicular, frozen ice masses exhibiting backscattering photometric behaviour²⁰. Over time, ice surfaces exposed to bombardment of cosmic rays, solar wind and micrometeorites should undergo analogous processes of comminution and agglutination that characterize regolith formation on the Moon²². Indeed, there is little reason to expect that regolith particles containing ice should be any less intricate in structure than their mechanically complex silicate counterparts in lunar soils, unless their temperature approaches the melting point.

An interesting alternative for producing intricate textures can be suggested based on laboratory experiments carried out by Johnson²³ who reported that layers of water seem to become more backscattering after bombardment by protons, apparently as a result of the formation of deep, micrometre-sized 'pits' in the surface layer. In this case the differences between the frosts on Earth and on outer satellites would be attributed to the effect of magnetospheric particle radiation of the surfaces of the outer satellites. □

TABLE 1 Photometric parameters for model endmembers and ice satellites

	Single-scattering albedo $\tilde{\omega}_0$	Asymmetry factor g
Ceres-type material ¹³	0.06	-0.4
Fresh terrestrial snow (actual) ¹²	0.999	+0.53
Backscattering snow (hypothetical)	0.999	-0.3
Mie sphere ($r=200\ \mu\text{m}$ at $\lambda=0.5\ \mu\text{m}$) ¹⁰	0.999	+0.89
Europa ²	0.97 ± 0.01	-0.15 ± 0.04
Mimas ²	0.93 ± 0.03	-0.30 ± 0.05
Enceladus*	0.98 ± 0.01	-0.46 ± 0.03
Rhea ³	0.891 ± 0.003	-0.321 ± 0.002
Miranda ⁴	0.54	-0.32
Ariel ⁴	0.72	-0.28
Oberon ⁵	0.41 ± 0.01	-0.29 ± 0.03
Umbriel ⁴	0.32	-0.31
Titania ⁴	0.48	-0.28

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Use of ^{10}Be in polar ice to trace the 11-year cycle of solar activity

J. Beer*, A. Blinov†, G. Bonani‡, R. C. Finkel§¶, H. J. Hofmann‡, B. Lehmann||, H. Oeschger||, A. Sigg||, J. Schwander||, T. Staffelbach||, B. Stauffer||, M. Suter§ & W. Wölfli‡

* Environmental Physics, Institute for Aquatic Sciences and Water Pollution Control, ETH-Zurich, c/o EAWAG, CH-8600 Dübendorf, Switzerland

† Department of Cosmic Research, Leningrad Polytechnic, Politechnicheskaya 29, Leningrad 195251, USSR

‡ Institute for Intermediate Energy Physics, ETH-Hönggerberg, CH-8093 Zürich, Switzerland

§ Paul Scherrer Institute, CH-5232 Villigen, Switzerland

¶ Physics Institute, University of Bern, CH-3012 Bern, Switzerland

A DETAILED knowledge of the history of solar magnetic activity is important in several respects. From satellite data there is increasing evidence that solar magnetic activity and solar irradiance are positively correlated, a conclusion with important implications for climatology¹. Because of the complexity of variations in solar activity, a long record is necessary for understanding the mechanisms responsible for heliomagnetic cyclicity². Here we show that ^{10}Be concentrations in polar ice can be used to study variations in the solar activity—particularly the 11-year cycle—in time periods pre-dating historical records.

The solar activity, expressed as the sunspot number, has been recorded consistently since 1843, when Schwabe discovered the 11-yr cycle. Earlier data can be reconstructed from historical documents^{3–5} containing references to auroral events as well as mentioning sunspot activity. Although much effort has been expended to extend the sunspot and auroral record further back, the data before 1600 become more and more incomplete and uncertain. One way to overcome this problem is to rely on indirect data such as records of cosmogenic radio-isotopes that are produced by the interaction of cosmic-ray particles with the atmosphere. From neutron flux measurements over the last few decades it is known that the cosmic-ray flux reaching the atmosphere is modulated by solar activity. Magnetic fields frozen in the solar wind deflect the flux of cosmic-ray particles with greatest effect at low energies, and lead to a reduction of the production rate of cosmogenic radionuclides. Although the details of the modulation process are not yet understood, there is a clear anticorrelation between solar activity and cosmic-ray flux reaching the Earth. Calculations^{6–9} reveal that variations of ± 20 –25% in the production of cosmogenic isotopes are to be expected for typical solar minima and maxima.

The two cosmogenic isotopes best suited to trace the solar activity are ^{14}C and ^{10}Be ^{10,11}. Both are produced by nuclear reactions with the principal components of the atmosphere but their fates are quite different. ^{14}C is oxidized to CO_2 and subsequently exchanges between the various terrestrial carbon reservoirs: atmosphere, biosphere and ocean. ^{10}Be , however, becomes fixed to aerosols and is removed from the atmosphere mainly by precipitation. The overall atmospheric residence time is ~ 1 –2 years¹². As a consequence of the different geochemical behaviour of ^{14}C and ^{10}Be , ^{14}C is well homogenized in the atmosphere and therefore reflects the mean global production rate much better than does ^{10}Be , which is strongly influenced by local transport and deposition processes. On the other hand, the large sizes and the long residence times of the carbon reservoirs lead to a strong damping of short-term production variations of ^{14}C . For the 11-yr cycle the attenuation factor for ^{14}C is ~ 100 (ref. 13) and for ^{10}Be it is ~ 1.25 . This damping makes detection of short-term variation with ^{14}C very difficult. More detailed discussions of ^{14}C variations and their relation to solar activity are given elsewhere^{14–16}. Despite the short-term fluctuations caused by transport and deposition effects, ^{10}Be turns out to be the most promising cosmogenic radio-isotope with which to detect the 11-yr Schwabe cycle¹⁰.

Although first detected in 1956^{17,18}, cosmogenic ^{10}Be only became a potential tool to reconstruct solar activity with the development of accelerator mass spectrometry (AMS) through which the detection sensitivity was increased by several orders of magnitude over decay counting^{19,20}.

Several studies have revealed higher ^{10}Be concentrations during extended periods of low solar activity (such as the Maunder Minimum)^{21–24} and indications of an 11-yr cycle in production^{25,26}. For a quantitative study of the 11-yr cycle, however, these records were limited either by the short period covered or by insufficient temporal resolution. In an earlier investigation we compared ^{10}Be concentrations with ^{10}Be fluxes to see which gave a better indication of production rate changes²³. That study showed no significant difference between fluxes and concentrations and we have therefore chosen to use concentrations here. The question should be reexamined when more detailed information about transport and deposition mechanisms are available.

We have analysed the upper part of a 300-m ice core to test whether ^{10}Be is a useful tool for determining past 11-yr-cycle solar activity. We did this by comparing ^{10}Be concentrations with directly observed parameters of solar activity such as the sunspot number and the Aa index (defined below). The core was recovered in 1986 at Dye 3, South Greenland (65.2° N; 43.8° W). To establish the timescale, a continuous record of H_2O_2 was first measured²⁷. The seasonal variations of H_2O_2 were then used to determine the individual annual layers. To confirm the absolute timescale, stratigraphic horizons were also used: the bomb peak of tritium in 1963 and the acidity peaks caused by the volcanic eruptions of Tambora (1815) and Laki (1783). Although minor uncertainties in the thickness of the annual layers cannot be excluded, the overall precision of the dating is within one year. The core was cut into annual samples with typical weights of 2 kg. The samples were then melted, 0.5 mg of ^9Be carrier added and the volume reduced by evaporation. Finally the $\text{Be}(\text{OH})_2$ was precipitated and converted to BeO . The measurements were carried out at the AMS facility of ETH/PSI²⁸.

The results are shown in Fig. 1 for the period 1783–1985. The experimental errors (1σ counting statistics) are in the range of 4–10%. To remove the rather large short-term fluctuations, most likely caused by deposition processes, a three-point binomial filter was applied to the measured ^{10}Be data (Fig. 1a). In Fig. 1b the filtered data are compared with the sunspot number. There is a clear anticorrelation between ^{10}Be and the sunspot number, both with respect to the 11-yr cycle and to the long-term trend, which may be considered as part of the 80-yr Gleissberg

¶ Permanent address: Nuclear Chemistry Division, Lawrence Livermore National Laboratory, Livermore, California 94550, USA.

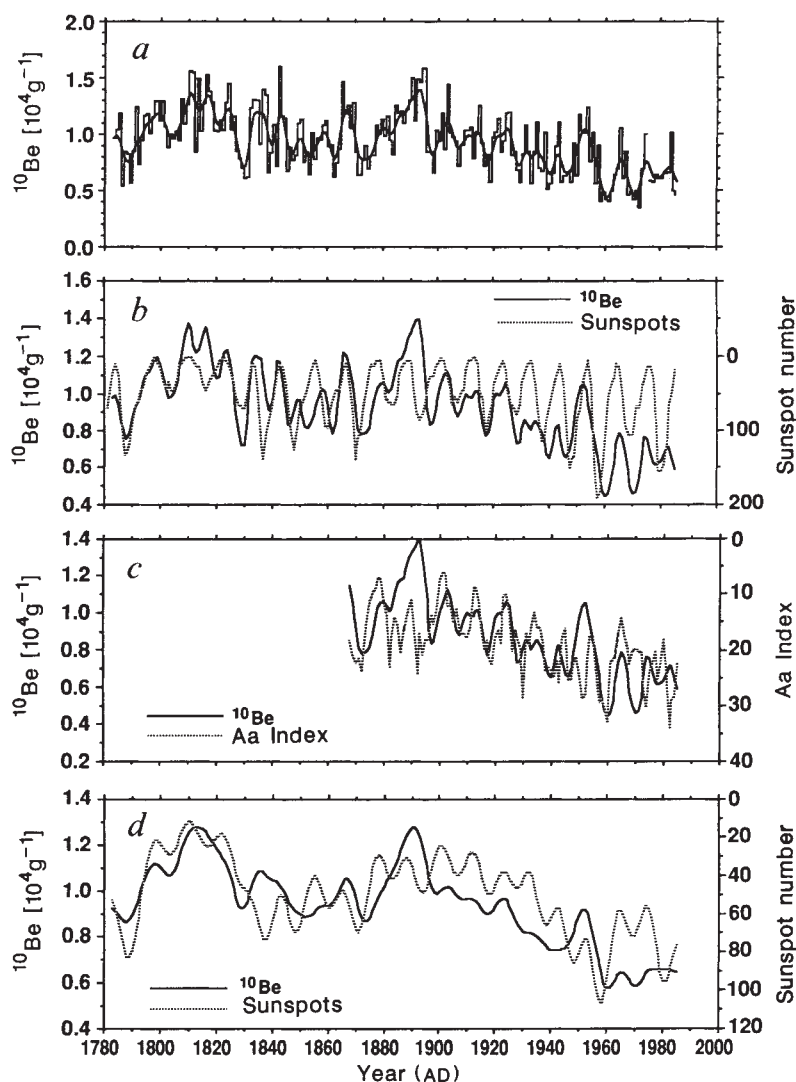
cycle²⁹. The main discrepancy is related to the fact that, whereas the minima in the sunspot number are always close to zero and therefore do not show much variation, the corresponding ^{10}Be maxima vary considerably. This indicates that sunspots and ^{10}Be are not identical indicators of overall heliomagnetic activity. The relationship breaks down especially at solar minimum, where few sunspots may occur in conjunction with considerable residual heliomagnetic activity. This can be seen by examining the Aa index, another indirect solar parameter, which is based on solar-wind-induced short-term (up to 3 h) variations of the geomagnetic activity measured at two antipodal stations^{15,30,31}. The Aa index has been recorded only since 1868. It agrees well with the ^{10}Be record, not only with respect to the 11-yr cycle but also reflects the decreasing trend of the ^{10}Be maxima and minima since ~1900 (Fig. 1c). In Fig. 2 the cross-correlation between the measured ^{10}Be data and the sunspot record is displayed as a function of lag. The highest anticorrelation is observed for a lag of ~1 year, which corresponds well with the mean atmospheric residence time of ^{10}Be . The cross-correlation with a significance of >99.99% proves the existence of the 11-yr cycle: the correlation factor changes periodically, reaching minima after lags of 11, 22, 33... $n \times 11$ years. The weakening of the correlation at long lags is due to the long-term trend which imposes a change in base line on the 11-yr variations. The cross-correlation curve between ^{10}Be and the Aa index is similar to the one between ^{10}Be and sunspots (Fig. 2) and is therefore not shown.

Another important question concerns the amplitude of the expected variations. As discussed elsewhere^{6,8,9} the global mean

production rate changes by about $\pm 25\%$ around the mean value between periods of sunspot minima and maxima. The amplitude observed in fallout at the Earth's surface will be damped by the ~1-yr residence time of ^{10}Be in the stratosphere. To calculate the variations in ^{10}Be to be expected at the latitude of Dye 3 one has to take into account the fact that solar modulation affects only stratospheric and not tropospheric production and that the stratospheric contribution to the total fallout is strongly latitude-dependent. Because this dependence is not yet understood in detail, the general approach is to use as a model the delayed ^{90}Sr fallout from bomb tests, which is transported from the stratosphere in a manner very similar to ^{10}Be (ref. 9). ^{90}Sr data from a large number of stations have been analysed in order to understand the distribution of atmospheric fallout^{32,33}. Based on the data compiled by Joseph *et al.*³² we find that the flux of ^{90}Sr at Dye 3 (65°N) is nearly equal to the mean global value. In equatorial regions and farther north the values are lower and at mid-latitudes the values are higher. The fluctuations from year to year are rather large and range from 60% to 120% of the mean. Combining the latitudinal distribution and variation of stratospheric input derived from ^{90}Sr and the attenuation factor owing to the atmospheric residence time (1.25, as calculated from a box model), with the estimated magnitude of the change in production owing to solar modulation ($\pm 25\%$), we find that the expected variations in ^{10}Be concentration at Dye 3 are in the range $\pm 15\text{--}35\%$.

As the measured data show rather large fluctuations from year to year, which are probably due to short-term variations in atmospheric transport and deposition processes, we believe

FIG. 1 Comparison of the ^{10}Be ice-core record with the sunspot number and Aa index. The experimental uncertainty (1σ counting statistics) is in the range of 4–10%. *a*, Measured ^{10}Be concentrations (step function). To remove short-term climate noise the data have been smoothed with a three-point binomial filter. *b*, Comparison of the smoothed ^{10}Be record with the annual sunspot number for the period 1783–1985. *c*, Comparison of the smoothed ^{10}Be record with the annual Aa index for the period 1868–1985. *d*, Comparison of the long-term trend (Gleissberg cycle) between ^{10}Be and the sunspot number. Both curves have been smoothed with a 19-point binomial filter.



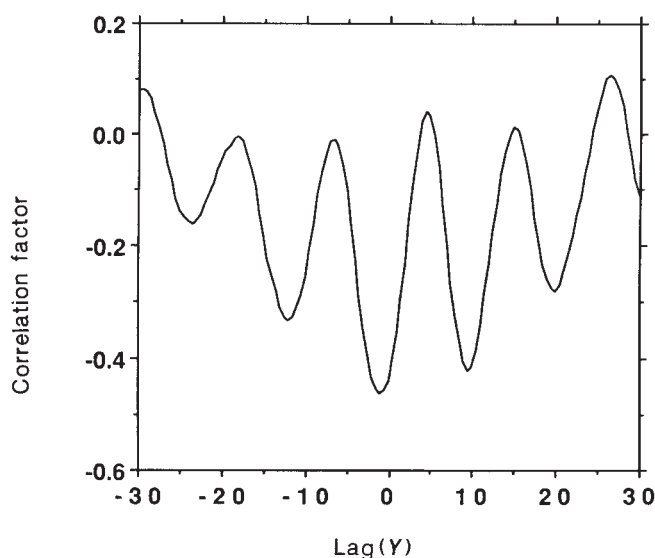


FIG. 2 Cross-correlation of the measured ^{10}Be data (Fig. 1a) with the sunspot number (Fig. 1b). The highest anticorrelation is reached at a time lag of ~ 1 year corresponding to the mean atmospheric residence time of ^{10}Be . The cross-correlation clearly shows the existence of the 11-yr Schwabe cycle.

that the comparison between measured and calculated amplitudes is best done with the smoothed ^{10}Be record of Fig. 1b. This smoothing introduces an attenuation factor of 1.28 in peak amplitudes. The mean relative amplitude for the 11-yr cycle from Fig. 1b has to be corrected for the attenuation generated by the smoothing. The resulting amplitude is $20\% \pm 15\%$. This value is well within the range of the calculated variations. There is evidence that meteorological effects influence the ^{10}Be concentration by sometimes amplifying the solar modulation signal and sometimes attenuating it. If there are any meteorological or climatological effects related to the 11-yr sunspot cycle, they seem to be of minor importance.

For easier comparison of the long-term trend both records have been filtered with a 19-point binomial filter and are displayed in Fig. 1d. Except for the past 20 years, the two curves show the same pattern of variation.

The data of Figs 1 and 2 show a clear anticorrelation between ^{10}Be and solar activity with the expected phase lag of ~ 1 year. The remaining discrepancy can be explained by fluctuations during the removal of ^{10}Be from the atmosphere and by the fact that solar modulation of cosmic rays, sunspot numbers and Aa indices are not identical representations of solar activity. Beside the shielding effects of the solar activity, there are a few other factors that may influence ^{10}Be production. These are solar cosmic rays, geomagnetic field intensity and anthropogenic effects. During periods of high solar activity, there is also a higher flux of solar particles reaching the atmosphere. Owing to the relatively high thresholds of the nuclear reactions leading to ^{10}Be , the contribution from these low-energy particles to the production rate of ^{10}Be is negligible (in contrast to the ^{14}C production)¹⁰. The geomagnetic dipole field also acts as a shield reducing the galactic cosmic-ray flux. During the past 200 years, however, the dipole part of the field was only slightly decreasing and the calculated effects on the ^{10}Be production are negligible³⁴. In contrast to ^{14}C , ^{10}Be is not influenced by anthropogenic effects such as the combustion of fossil fuels or nuclear-bomb tests^{10,11}.

The main uncertainty in the interpretation of ^{10}Be data in terms of solar activity modulation is therefore the extent to which fluctuations induced by 'climate noise' (atmospheric mixing, precipitation rate, scavenging efficiency) affect the polar ice cores that we have studied. Possible ways to correct for these climate effects are to search for normalization parameters and to combine records from different sites (such as the Antarctica and the Arctic) with different climate conditions but similar production rates^{24,35}.

Our study proves that ^{10}Be in polar ice is a useful tool to extend significantly the historical record of solar activity, provided an appropriate timescale is available. This opens up the possibility of studying the long-term behaviour of solar activity

and the history of solar-terrestrial relationships. In particular several interesting questions can be addressed such as: (1) Is the Schwabe cycle driven by processes in the convection zone or by a precise chronometer deep in the Sun³⁶? (2) How does the heliomagnetic activity behave during periods of solar sunspot minima (such as the Maunder and Spörer minima) with respect to periodicity, amplitude and phase? (3) How did the solar activity change in the past and how does the climate system respond to such changes³⁷? □

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Weak-link-free behaviour of high-angle $\text{YBa}_2\text{Cu}_3\text{O}_{7-\delta}$ grain boundaries in high magnetic fields

S. E. Babcock*, X. Y. Cai*, D. L. Kaiser‡
& D. C. Larbalestier*†

* Applied Superconductivity Center and

† Department of Materials Science and Engineering,
University of Wisconsin, 1500 Johnson Drive, Madison,
Wisconsin 53706, USA

‡ Ceramics Division, National Institute of Standards and Technology,
Gaithersburg, Maryland 20899, USA

A CRUCIAL issue in both the physics and the application of high-temperature superconductors is the low transport critical current density (J_{ct}) of polycrystalline materials. Much thinking about this issue has been defined by the thin-film $\text{YBa}_2\text{Cu}_3\text{O}_{7-\delta}$ bicrystal experiments of Dimos *et al.*^{1,2}, which clearly showed greatly reduced values of J_{ct} at high-angle grain boundaries (misorientation angle $\geq 10^\circ$). The reproducibility of this result for a wide range of misorientation relationships led them to conclude that all high-angle grain boundaries act intrinsically as Josephson junctions. By contrast, we describe here two high-angle grain boundaries with superconducting properties that clearly lack the weak-link signatures characteristic of Josephson junctions. Our bulk-scale bicrystal results provide direct evidence that not all high-angle grain boundaries are alike in their superconducting properties, and that at least some high-angle boundaries can carry significant supercurrents at 77 K in high magnetic fields.

Crystallographic and electrical transport characteristics of five flux-grown³ bicrystals and one single crystal were investigated. The bicrystals were composed of square-plate grains that were 125–375 μm on a side and 50–180 μm thick. Thus, the grain boundaries were on a bulk scale. Bicrystal 1's crystals were rotated relative to one another by $\sim 90^\circ$ about a nearly common b axis. (Owing to the twin microstructure, the a and b axes are not distinguished.) Light-microscopy trace analysis (LMTA) indicated that the boundary was composed largely of two planar facets with the geometry illustrated in Fig. 1. Adjacent crystals in bicrystals 2–5 were related by rotations of 3° , 14° , 22° and 38° , respectively, about their nearly common ($\leq 3^\circ$ misorientation) c axes. These latter four misorientation relationships are in the same family as the thin-film bicrystals originally probed

by Dimos *et al.*¹. Whereas the thin-film boundaries were nominally symmetrical tilt in character, LMTA suggested that the present bulk-scale boundaries were composed largely of mixtures of asymmetrical tilt-, twist- and mixed-type planar facets. (See ref. 4 for definitions of boundary types.)

Gold wire leads were attached with Ag epoxy in a four-terminal configuration. For each bicrystal, the zero-field resistance fell sharply between 93 and 92 K. Two distinctly different types of behaviour, however, were observed at the low-resistance end of the transition. Figure 2 shows the transitions for bicrystal 1 with the magnetic field H parallel to the c_1 axis, $H \parallel c_1$ (see Fig. 1). These curves clearly indicate a rapid decrease to zero resistance at all fields up to 7 T. Furthermore, the voltage-temperature (V - T) characteristics are essentially the same as those observed previously for $\text{YBa}_2\text{Cu}_3\text{O}_{7-\delta}$ single crystals⁵. Evidence for similar behaviour was found for bicrystals 2 and 3 and for one of the single crystals in bicrystal 4, which all showed a single, very sharp transition to zero resistance ($V < 10$ nV). By contrast, residual resistance, the detailed characteristics of which depended on the measuring current (~ 10 A cm^{-2}), was observed at the low-temperature end of the transition for bicrystals 4 and 5. In bicrystal 5 the transition occurred in two sharp steps. The first step occurred at 93 K and the second at 86 K and 79 K for measuring current densities of ~ 12 A cm^{-2} and ~ 4 A cm^{-2} , respectively. For bicrystal 4, a low-resistance tail to zero resistance at 86 K was found at 0 T and ~ 2 A cm^{-2} . At 0.5 T this tail was broadened such that zero resistance was not obtained at $T \geq 77$ K.

The J_{ct} - H characteristics at 77 K of bicrystals 1–3 and of one single crystal in bicrystal 4 were measured in fields up to 7 T. J_{ct} was defined at a voltage of 1 μV . For bicrystals 2 and 3 and for the single crystal, H was aligned parallel to the common c axis ($H \parallel c$). Bicrystal 1 was tested in three configurations: $H \parallel c_1$, $H \parallel c_{11}$, and $H \perp c_1, c_{11}$ (high- H_{c2} , high upper critical field, orientation for both crystals). The results (Fig. 3) have several important features. All voltage-current (V - I) traces exhibited the smooth onset of dissipation without hysteresis typical of flux flow. The general form of the J_{ct} - H characteristic of all three bicrystals and the single crystal was very similar for $H \parallel c$. The magnitude of J_{ct} at 5 T varied from 400 A cm^{-2} (14° bicrystal) to 1,800 A cm^{-2} (3° bicrystal). The J_{ct} values for the single crystal fell between those of the 3° and 90° bicrystals. For all cases except bicrystal 1 with $H \perp c_1, c_{11}$, J_{ct} - H showed a smooth decrease towards zero at fields near 7 T. This applied field is typical of the irreversibility field for the $H \parallel c$ orientation⁶. For $H \perp c_1, c_{11}$ in bicrystal 1, the J_{ct} - H characteristic was much flatter

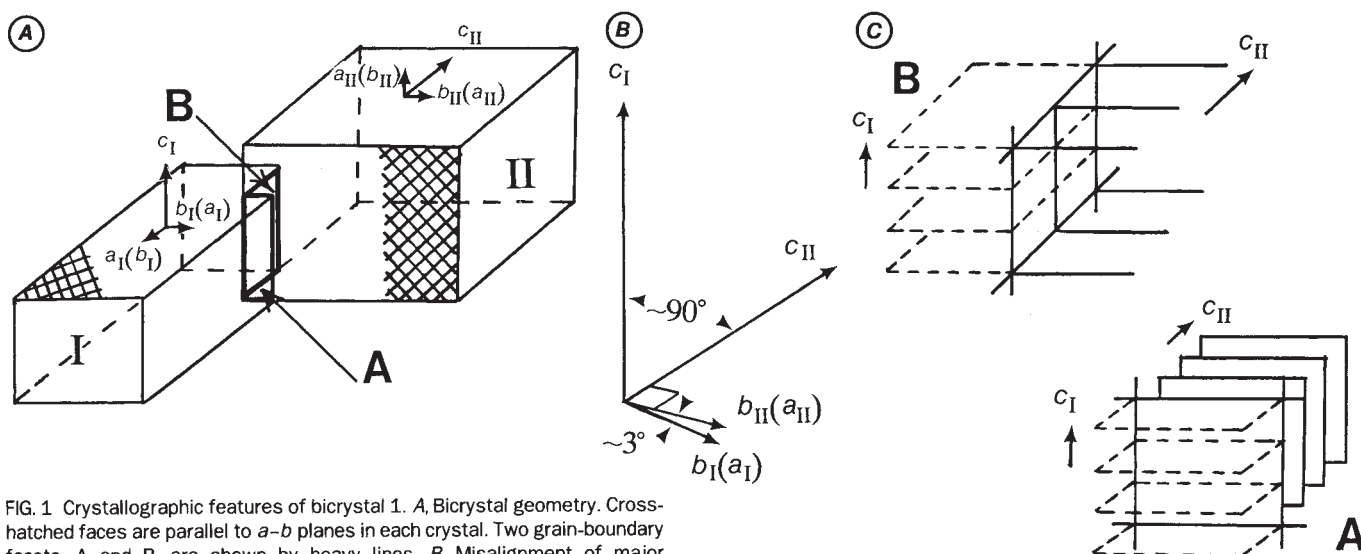


FIG. 1 Crystallographic features of bicrystal 1. A, Bicrystal geometry. Cross-hatched faces are parallel to a - b planes in each crystal. Two grain-boundary facets, A and B, are shown by heavy lines. B, Misalignment of major crystallographic axes. C, Orientation of grain-boundary plane relative to a - b planes for facets A and B. Dotted lines represent planes in crystal I.

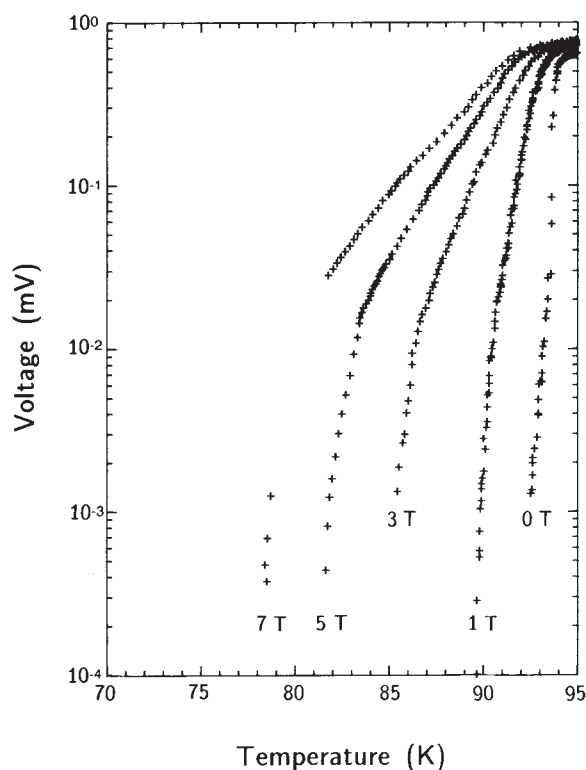


FIG. 2 Voltage as function of field and temperature across the grain boundary of bicrystal 1. Field was applied parallel to c_1 (see Fig. 1). Measuring current was 10 mA.

at 7 T, as is expected for this high- H_{c2} orientation. These observations are consistent with the conclusion that both the single crystal and the bicrystal J_{ct} - H characteristics are controlled by flux-pinning processes.

This conclusion was explored further by measuring J_{ct} in very weak fields of 0–30 mT at 77 K for bicrystal 1 ($H \parallel c_{II}$, Fig. 4),

bicrystal 3 ($H \parallel c$) and for the single crystal in bicrystal 4 ($H \parallel c$). No dependence of J_{ct} on field was found for these bicrystals or for the single crystal. In addition, there was no asymmetry in J_{ct} with respect to the sign of the current and no hysteresis in the V - I traces. By contrast, J_{ct} asymmetry, V - I hysteresis, and sensitivity of J_{ct} to fields < 1 mT at 4.2 K were observed for the thin-film bicrystals and were presented as evidence of the Josephson junction character of the high-angle grain boundaries². The properties of bulk-scale high-angle bicrystals 1 and 3 contrast strongly with those of the thin-film bicrystals in all these respects. Their properties do not show Josephson junction characteristics, but rather behaviour that is indicative of a J_{ct} controlled by flux pinning.

In considering the more general implications of our results, we note that bicrystals 2–5 fall in the range originally studied in thin films¹. Dimos *et al.* identified two regimes of behaviour: (1) boundaries with very small misorientations ($\leq 5^\circ$) that had little effect on the J_{ct} value and (2) boundaries with misorientation angles $\geq 10^\circ$ that had strongly diminished J_{ct} values and showed Josephson junction characteristics^{1,2}. Although the behaviour of bicrystals 2, 4 and 5 is qualitatively consistent with their conclusions, bicrystals 1 and 3 show quite different behaviour than the previous work would predict. Bicrystal 3 (14° boundary) should belong to category (2) above, yet its high-field and low-field J_{ct} - H properties are not characteristic of Josephson junctions. This is also the case for bicrystal 1. No directly comparable data for boundaries with misorientation angles approaching 90° about a common b axis is available from the thin-film bicrystal experiments, but the conclusions of Dimos *et al.*^{1,2} imply inclusion of this boundary in the high-angle Josephson junction regime.

The weak-link-free character of bicrystal 1 is particularly striking. With respect to the anisotropy of the electrical properties, the misorientation relationship of this bicrystal must be considered the highest possible, because it causes the copper-oxygen planes of crystal I to be almost perpendicular to the copper-oxygen planes of crystal II. Furthermore, basal-plane-faceted boundaries, such as facet A, have been argued to be most detrimental to J_{ct} ⁷. But the V - T and J_{ct} - H characteristics of

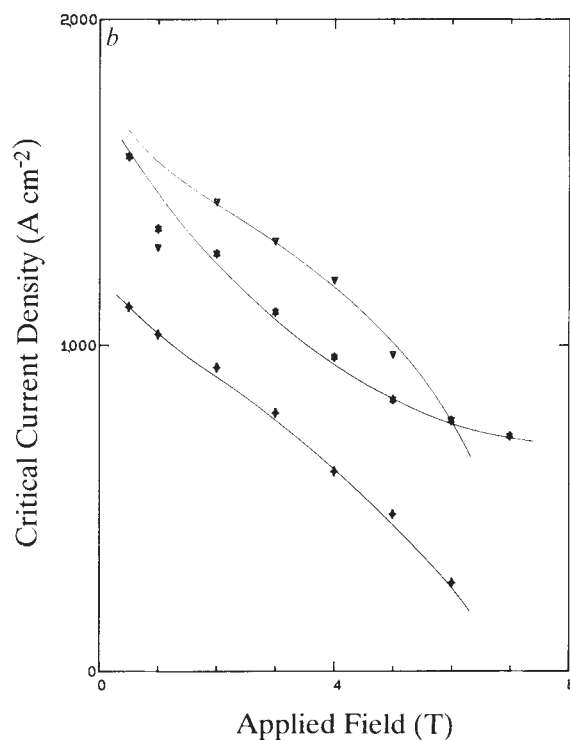
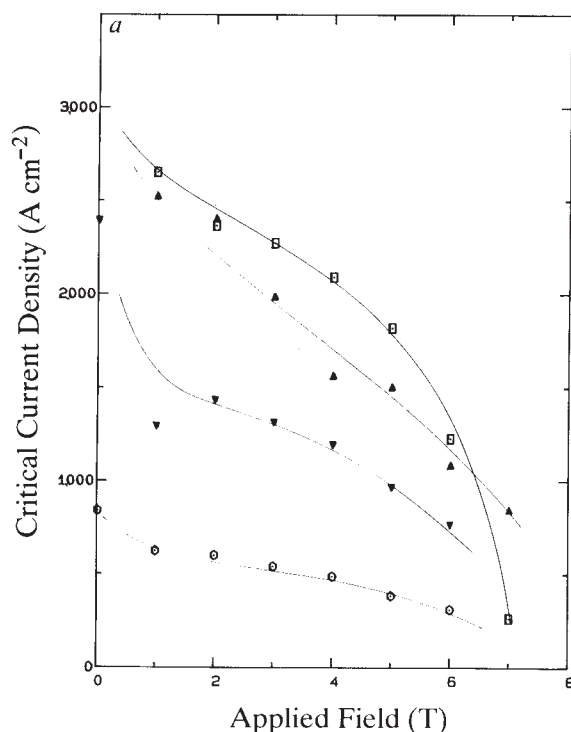


FIG. 3 *a*, J_{ct} - H characteristics with $H \parallel c$ for bicrystals 1 ($\blacktriangledown$), 2 ($\square$) and 3 ($\circ$) at 77 K and for intra-grain region of single crystal in bicrystal 4 ($\blacktriangle$) at 73 K.

b, J_{ct} - H characteristics for bicrystal 1 in $H \parallel c_1$ ($\blacktriangledown$), $H \parallel c_{II}$ ($+$) and $H \perp c_1, c_{II}$ ($\star$) orientations.

bicrystal 1 are largely the same as those measured for the single crystal. These results are consistent with the high J_{ct} (77 K and 0 T) obtained for a thin-film track that contained several boundaries of a similar misorientation relationship⁸. In contrast to our measurements, however, no information about the nature of the coupling across the thin-film boundary is provided by the isolated zero-field measurement of Chan *et al.*

There are several ways in which flux-grown bicrystals may differ from their epitaxial thin-film bicrystal counterparts. Among these, the most important may be the tendency for flux-grown bicrystals to self-select low-energy configurations as they grow⁹, rather than to assume crystallographies predetermined by a substrate^{1,2}. These low-energy configurations seem to correlate with near-coincident-site lattice (NCSL) misorientations that are characterized by relatively low Σ values^{7,9}. (For the case of exactly coincident lattice sites, $\Sigma^{-1} \equiv$ fraction of lattice sites in one crystal that are coincident with lattice sites in the other crystal. Because of the orthorhombic lattice, the low-energy misorientations for $\text{YBa}_2\text{Cu}_3\text{O}_{7-\delta}$ are associated with NCSL misorientations. Σ is used in the same way to describe NCSL misorientations.) It is possible that the superconducting properties of the boundary correlate with the Σ value and/or the deviation from the exact low-energy misorientation, $\Delta\omega$ ^{7,10}. Even though bicrystals 1–5 can be described as near- $\Sigma=3$, near- $\Sigma=1$, near- $\Sigma=41$, near- $\Sigma=13$ and near- $\Sigma=5$ NCSL misorientations, respectively, with varied and in some cases large $\Delta\omega$ values, our few experiments do not provide conclusive evidence concerning the importance of the Σ or $\Delta\omega$ value. In fact, there are clearly a number of other possible crystallographic and processing-related origins for the observed variety in properties to be investigated in future experiments. For example, the properties may also be influenced by the boundary plane, the detailed character of segregation^{11,12}, or the presence of impurities¹⁶ in complicated and interconnected ways. The variety of factors noted here emphasize that many aspects of grain boundaries must be investigated before their intrinsic properties can be defined unambiguously.

Although the reasons for this different behaviour are unclear, the discovery of high-angle grain boundaries that are not Joseph-

son junctions is important. This conclusion may be overlooked if emphasis is placed only on the magnitude of the zero-field J_{ct} , because the thin-film bicrystals^{1,2} still have significantly larger $J_{ct}(H=0)$ values than our bulk-scale bicrystals. But, in contrast to the bulk-scale bicrystals, the thin-film bicrystals show the strong undesirable decrease of $J_{ct}(H)$ in small (milliTesla) fields that is observed in polycrystalline materials¹³ and which is characteristic of Josephson junctions. By contrast, bulk-scale high-angle bicrystals 1 and 3 show evidence of a $J_{ct}-H$ behaviour that is controlled by flux pinning. To develop useful J_{ct} properties in larger magnetic fields this transition from a Josephson junction to a flux-pinning grain-boundary characteristic must be made. An important issue is to determine whether techniques reported to raise the intra-grain J_{ct} of bulk samples to 10^5 – 10^6 A cm^{-2} at 77 K (for example, second-phase pinning¹⁴ and neutron irradiation¹⁵) are also effective for raising the inter-grain $J_{ct}-H$ value. □

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Assessment of urbanization effects in time series of surface air temperature over land

P. D. Jones*, P. Ya. Groisman†, M. Coughlan‡, N. Plummer‡, W.-C. Wang§ & T. R. Karl||

* Climatic Research Unit, School of Environmental Sciences, University of East Anglia, Norwich NR4 7TJ, UK

† State Hydrological Institute, Leningrad, USSR

‡ Bureau of Meteorology, Melbourne, Australia

§ Atmospheric Sciences Research Center, State University of New York, Albany, New York 12205, USA

|| National Climate Data Center, Asheville, North Carolina 28801, USA

RECORDS of hemispheric average temperatures from land regions for the past 100 years provide crucial input to the debate over global warming^{1–4}. Despite careful use of the basic station data in some of these compilations of hemispheric temperature^{1,2,4–6}, there have been suggestions^{7,8} that a proportion of the 0.5 °C warming seen on a century timescale may be related to urbanization influences—local warming caused by the effects of urban development. We examine here an extensive set of rural-station temperature data for three regions of the world: European parts of the Soviet Union, eastern Australia and eastern China. When combined with similar analyses for the contiguous United States^{9,10}, the results are representative of 20% of the land area of the Northern Hemisphere and 10% of the Southern Hemisphere. The results show that the urbanization influence in two of the most widely used hemispheric data sets^{1,2,4} is, at most, an order of magnitude less than the warming seen on a century timescale.

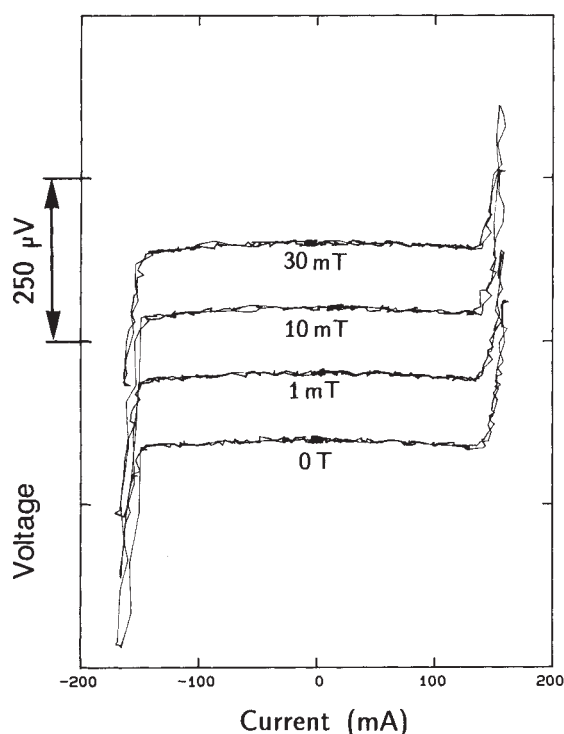


FIG. 4 $V-I$ traces for bicrystal 1 in weak applied fields at 77 K for $H \parallel c_{II}$ (see Fig. 1).

Significant urbanization effects have been noted at many cities^{7,11}. Two factors must be considered, however, when comparing individual city 'heat island' magnitudes with hemispheric warming trends. First, many of the extreme urban biases that have been quoted are the largest daily occurrences, perhaps happening during still evenings or intense inversions. The effect on station monthly mean temperatures is sure to be considerably smaller. Second, in any gridded temperature data set, a single affected station is unlikely to have a large influence on the time series of the nearest grid-point, because this is generally a weighted average of between 5 and 20 station records^{1,2}.

The most comprehensive attempt to assess the significance of urbanization on large-scale temperature trends has been made for the contiguous United States by Karl *et al.*¹². In this analysis the Historical Climate Network (HCN)¹³ of 1,219 stations was used to assign stations to pairs of rural and urban sites. Karl *et al.* showed that an urbanization influence could be detected in many records, with the urban bias being a nonlinear function of population. To assess the influence of urbanization on regional averages developed from the gridded data sets¹⁻³, the average for the contiguous United States derived from the HCN data set of principally rural stations was compared with the gridded series. The gridded series make use of some of the temperature data that have been assembled over the present century from sources such as World Weather Records and Monthly Climatic Data for the World (see ref. 5 for details of these sources). Some of these records come from large cities and could be affected by urbanization-related trends. Comparison of the gridded and the HCN series revealed that the gridded time series shows a warming of $\sim 0.1^\circ\text{C}$ over the period 1901-84⁹. It can be argued¹⁰, however, that the rate of urbanization growth in the United States is atypical compared with many other parts of the world. We have therefore attempted to assess the urbanization influence in other regions of the world using specially developed rural-station temperature series.

Western Soviet Union. For the western part of the Soviet Union we selected a network of 38 stations from sites in non-urbanized areas with long records (Fig. 1a). The sites include isolated meteorological stations, lighthouses, villages and other small settlements. The largest populated sites are nine towns with populations of the order of 10,000 people. All the site records were assessed for artefacts due to factors such as site moves or changing methods used to calculate monthly mean temperatures. At twelve sites the observing station was moved slightly. Comparisons with neighbouring sites were made before and after each change, and where necessary, corrections were made to ensure homogeneity of the rural-station record. No corrections were deemed necessary for the remaining 26 stations, where no station moves were reported.

Data from the 38 stations were then converted to anomalies from the 1951-75 average and combined using the inverse-distance weighting gridding scheme used in ref. 1. By using the same averaging scheme as for the gridded data, any differences between this average and the gridded average will be the result of data differences and not due to differing interpolation methods. The resulting series (RUSSR, Fig. 2a) is an average for the European part of the Soviet Union, parts of western Siberia and Kazakhstan.

Using the gridded data from ref. 1, we developed a regional time series (JUSSR) for the region using 22 gridpoints (see Fig. 1a). Similarly, we developed a regional time series (VUSSR) for this region using the hemispheric data set from ref. 4. An optimum interpolation procedure was employed by Vinnikov *et al.*⁴ to produce their annual hemispheric average time series, using about one-third the number of stations that were used in ref. 1. In this procedure, interpolation of station data to grid points was not undertaken, the published data being available only as annual hemispheric averages. For our study, regional time series were constructed from the data of ref. 4 using this

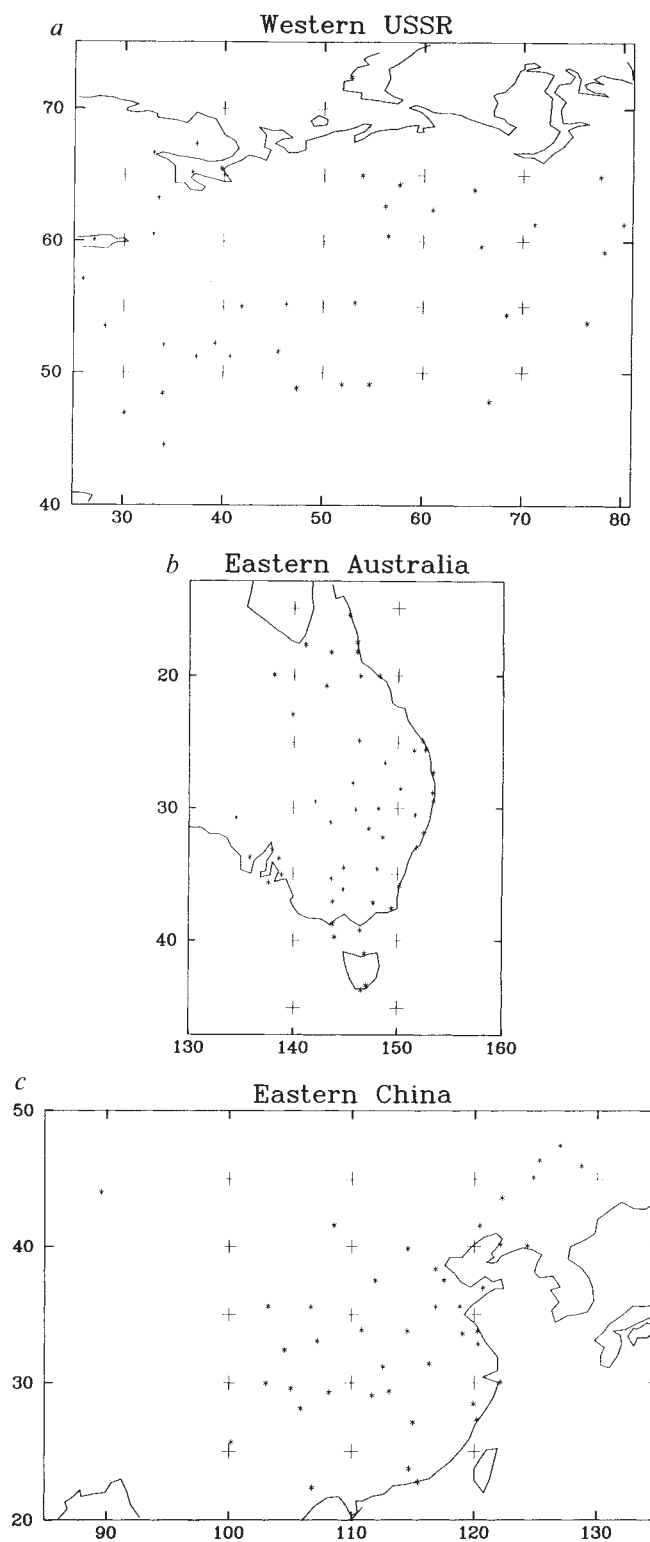


FIG. 1 Study regions, showing the rural stations (*) and the grid points (+). a, Western USSR; b, Eastern Australia; c, Eastern China. Details of the rural station networks are included in the text. The grid points are taken from the data set of refs 1 and 2, which interpolate station data onto a 5° latitude by 10° longitude grid. Station data used in the gridding extend 2.5° of latitude and 5° of longitude away from each grid point. For the western USSR, 60 station records were used to construct the grid-point series. There were 25 stations in operation by 1901 and 32 were operating in 1987. For eastern Australia 20 stations were used, 7 of which were operating during the 1930s and 15 of which were operating in 1988. For eastern China 38 stations were used, all of which were in operation in 1954, whereas only 29 were still operating in 1983. For the western USSR region only, there are four stations common to the grid-point and rural time series.

same optimum interpolation scheme. The stations used extend beyond the bounds of the rural network, unlike those used in ref. 1.

The rural-network average was compared with the two gridded data series over two periods, 1901–87 and 1930–87. Inter-correlation between the three series over these two periods is exceedingly high, with all correlations between 0.98 and 0.99. Comparisons of the standard deviation and linear trend are given in Table 1. The similarity between the statistics for all three series over both periods is remarkable. Over the 1930–87 period, a cooling of $\sim 0.2^\circ\text{C}$ in RUSSR is observed. This cooling is about 0.1°C smaller in JUSSR, but there are no statistically significant differences between the two series. Given that all three data sets make use of different station networks, slight differences between the time series and their trends are to be expected.

Eastern Australia. We assembled a network of 49 stations in the eastern half of Australia, from the states of Queensland, New South Wales, Victoria and the southeastern quarter of South Australia (Fig. 1b). All stations are rural or small village sites and all encompass the period 1930–88. The average population of the stations is 5,775; the maximum population is 33,368 and there were seven lighthouse sites which were assumed to have a negligible population. The series from all stations were reduced to anomalies from the 1951–80 period before being combined into a regional series (RAUS, Fig. 2b) using the same inverse-distance gridding scheme.

A regional time series (JAUS) was developed for this region from ref. 2, using grid points (Fig. 1b). We also used the data in ref. 4 to derive a regional series (VAUS) for the region, which incorporated between 7 and 27 stations.

The correlation coefficients between the RAUS, JAUS and VAUS series over the 1930–88 period are all between 0.95 and 0.96. Comparisons of the standard deviation and linear trend are shown in Table 1. The results indicate that neither JAUS nor VAUS differ significantly from the rural time series RAUS. For VAUS, the warming over the region is similar to that from the rural series. In all three series, warming over the 59-year period is statistically significant at the 5% level.

The rural data set for Australia can be split into time series based on maximum and minimum temperatures. Calculation of trends for these series shows that the 1930–88 warming is greater for the minimum temperatures (a 0.81°C rise) than for the maximum temperatures (a 0.30°C rise). This result indicates a

reduction in the daily temperature range from this region, a feature noted for the United States by Karl *et al.*¹⁴. It is unfortunate that separate maximum and minimum temperature data sets are not more widely available. Many countries calculate mean monthly temperatures using observations from a number of fixed hours per day, without recording the daily maximum and minimum temperatures.

Eastern China. We assembled a network of 42 station pairs of rural and urban sites in the eastern half of China (Fig. 1c). The data cover the period 1954–83. The 84 stations were selected from a 260-station temperature set recently compiled under the US Department of Energy and People's Republic of China Academy of Sciences Joint Project on the Greenhouse Effect¹⁵. The stations were selected on the basis of station history: we chose those with few, if any, changes in instrumentation, location or observation times. All 84 records were complete for the 30-year period. The urban stations were in regions with populations of over 0.5 million, whereas for the rural stations populations mostly less than 0.1 million (according to 1984 population statistics). From the 42-station rural network, we formed an average (RCHI, Fig. 2c) for eastern China using the inverse-weighting gridding scheme. The 42-station urban network (UCHI) was averaged in the same way. Using the gridded data from ref. 1, a regional time series (JCHI) was developed for the region encompassing 15 grid points (see Fig. 1c). An average series (VCHI) for the region, making use of 32 stations, was developed from the data in ref. 4.

The correlation between the RCHI and JCHI series over the 1954–83 period is 0.90, and there is a lower correlation of 0.81 between RCHI and VCHI. Comparisons of the standard deviation and linear trend are shown in Table 1. Although the results indicate that both gridded series show a slight cooling relative to the Chinese rural network (RCHI) over the 30-year period, differences between all three series are not statistically significant. Slight differences are to be expected given that the various series make use of different sets of raw station data. The

TABLE 1 Comparison of temperature trends

Series	Period	Standard deviation ($^\circ\text{C}$)	Linear trend ($^\circ\text{C}$ over period)
Western USSR			
RUSSR (rural)	1901–1987	0.82	0.38
JUSSR	1901–1987	0.79	0.38
VUSSR	1901–1987	0.81	0.31
RUSSR (rural)	1930–1987	0.84	–0.21
JUSSR	1930–1987	0.82	–0.09
VUSSR	1930–1987	0.85	–0.20
Eastern Australia			
RAUS (rural)	1930–1988	0.31	0.56*
JAUS	1930–1988	0.31	0.60*
VAUS	1930–1987	0.34	0.55*
Eastern China			
RCHI (rural)	1954–1983	0.30	0.23
JCHI	1954–1983	0.27	0.19
VCHI	1954–1983	0.37	0.13
UCHI	1954–1983	0.32	0.39*
Contiguous United States			
Rural ^{9,10}	1901–1984	0.42	0.16
Grid ^{9,10}	1901–1984	0.39	0.31

* Significant trend at the 5% level.

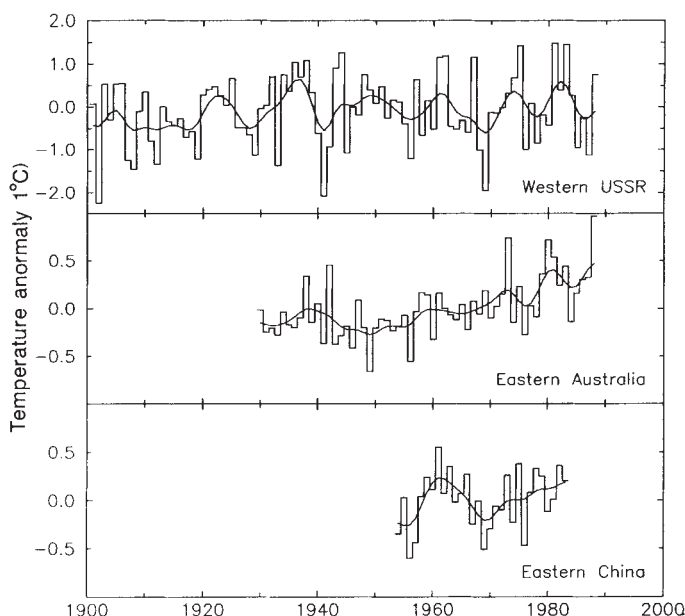


FIG. 2 Time series of annual temperature anomalies for the three regions: a, Western USSR (1901–88); b, Eastern Australia (1930–88); c, Eastern China (1954–83). The smooth curves were obtained using a gaussian filter designed to suppress variations on timescales of less than 10 years. The base periods for the three regions are 1951–75, 1951–80 and 1954–83, respectively. For the Chinese region all the rural stations are available for every year. For Eastern Australia, 3% of the annual station values are missing. For the Soviet series, there were only 20 of the 38 stations contributing in 1901. Seven stations began recording after 1930. Over 1930–87, the number of missing values amounts to 8% of the total. Most of these missing values were between 1930 and 1945.

warming in UCHI is 0.39 °C, considerably higher than that in RCHI. For this region, UCHI is the only series for which warming is statistically significant. The Chinese results may, at first glance, seem somewhat surprising, as 24 of the stations used in ref. 1 are among the 42 urban sites. In refs 1 and 4, average temperatures for these sites generally come from airport sites, which may be in rural areas. In many parts of the world, city-centre observatories were relocated to airport sites during the 1950s and 1960s. That temperature records from many sites are a combination of information from various locations within a city or town emphasizes the need to assess the homogeneity of individual site records^{5,6}.

In none of the three regions studied here is there any indication of significant urban influence in either of the two gridded series^{1,2,4} relative to the rural series. Earlier work on the contiguous United States^{9,10} showed an urban influence of 0.15 °C over the period 1901–84. (The results of this work are summarized in Table 1.) The United States result therefore does seem to be somewhat atypical compared with other industrialized regions of the world. The results from the United States clearly represent an upper limit to the urban influence on hemispheric temperature trends.

In total, the three regions and the contiguous United States encompass 82 grid points (22 over the western USSR, 14 over eastern Australia, 16 over eastern China and 30 over the contiguous United States) on a resolution of 5° latitude by 10° longitude. The three Northern Hemisphere regions in total comprise about 20% of the landmass of the hemisphere, and the eastern Australian region comprises 10% of the landmass in the Southern Hemisphere. Thus there seems to be little urbanization influence in three regions of the world that, when taken together, are twice the size of the contiguous United States.

It is unlikely that the remaining unsampled areas of the developing countries in tropical climates, or other highly populated parts of Europe, could significantly increase the overall urban bias above 0.05 °C during the twentieth century. A bias of this order is an order of magnitude smaller than the hemispheric and global-scale warming trend observed over the last 100 years. The bias will be further halved in hemispheric and global temperature estimates that incorporate marine as well as land temperatures.

We emphasize, however, that our results do not imply that urban warming influences in observations of local temperature will remain inconsequential in global averages in the future. Indeed, careful selection, inspection and monitoring for urbanization influences in the climate record will be required. This concern could be greatly lessened by an international effort to monitor and place observing stations outside urban areas. □

Identification of a deep marine source of particulate organic carbon using bomb ¹⁴C

Ellen R. M. Druffel* & Peter M. Williams†

* Woods Hole Oceanographic Institution, Woods Hole, Massachusetts 02543, USA

† Scripps Institution of Oceanography, University of California at San Diego, La Jolla, California 92093, USA

THE influx of bomb radiocarbon (¹⁴C) into the oceanic food chain has been evaluated by radiocarbon dating of pelagic organisms in the North Pacific^{1,2}. These studies found a significant gradient with depth of $\Delta^{14}\text{C}$ (the per mil deviation from the 'standard' activity of nineteenth century wood). Such a gradient is not expected according to long-standing assumptions about carbon cycling in the water column^{3–5}; instead, one could expect the $\Delta^{14}\text{C}$ of organisms throughout the water column to have become equal to that in surface-water dissolved inorganic carbon (DIC) by about 1970 (10–20 years after the production of bomb radiocarbon). Here we present $\Delta^{14}\text{C}$ values measured in the profiles of suspended and sinking particulate organic carbon (POC) from an open-ocean site and a coastal basin. The ¹⁴C activity of suspended POC decreases significantly with depth, as is observed in organisms, whereas that of sinking POC is only slightly lower than that in surface DIC and surface suspended POC. All POC $\Delta^{14}\text{C}$ results, however, are greater than the corresponding pre-bomb, surface-derived DIC values, and therefore contain bomb ¹⁴C. This decrease in ¹⁴C activity requires a deep source (or sources) of carbon to sub-surface POC pools. Adsorptive processes involving low-¹⁴C-activity dissolved organic carbon (DOC) may provide a mechanism for lowering $\Delta^{14}\text{C}$ values in suspended and (to a lesser extent) sinking POC.

$\Delta^{14}\text{C}$ of POC and DOC was measured in samples collected from two areas in the North Pacific Ocean. An oligotrophic site (31°00' N, 159°00' W) in the North Central Pacific (NCP) was occupied during June–July 1987 (bottom depth 5,760 m) and a series of profiles was taken from May 1986 to October 1987 in the centre of the Santa Monica Basin (SMB), a moderately eutrophic basin off the coast of California (33°50' N, 118°50' W, bottom depth 900 m). Suspended POC was collected using *in situ* pumps⁶ deployed for 2–8 hours at eleven depths in the North Central Pacific during 1987 and for 2–6 hours at four depths in the Santa Monica Basin during October 1986. During each deployment, 600–4,500 l of sea water were filtered through 0.8- μm pore-diameter, pre-combusted quartz-fibre filters (Whatman ultra-pure QM-A), which were then frozen at –20 °C. Sinking POC was collected at 600 m and 1,600 m from the bottom (5,165 and 4,165 m depth) in the North Central Pacific and at 100 or 200, 500, 700 and 850 m depths in the Santa Monica Basin using paired Soutar traps⁷ deployed for periods of two weeks (North Central Pacific) and three months (Santa Monica Basin). Mercuric chloride was used in all deployments as a trap poison. Sinking POC in the North Central Pacific samples was concentrated by filtration onto pre-combusted quartz filters. The filters were acidified with 5% H₃PO₄ for 24 h to remove the carbonates, dried under vacuum and combusted in double quartz tubes with CuO and silver according to standard techniques⁸. Aliquots of the sinking POC from SMB samples were acidified with 5% H₃PO₄ and dried at 60 °C before combustion as above. The Santa Monica Basin seawater samples were filtered on board ship from a 12-l Go-Flo Niskin bottle, and DOC was oxidized in the laboratory to CO₂ by photo-oxidation using previously reported techniques⁹. DIC was extracted from 150-ml aliquots (SMB) of the above unfiltered Niskin samples after acidification with 5% H₃PO₄, or absorbed into SrCl₂–NH₄OH solution on board ship from 200 l of the NCP Gerard

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barrel samples after acidification with concentrated H_2SO_4 (ref. 10). These latter CO_2 samples were measured by conventional gas proportional-counting techniques¹¹. The CO_2 from DIC (SMB), DOC and POC were converted to graphite¹², and ^{14}C was measured using accelerator mass spectrometry (AMS) at the University of Arizona¹³.

The suspended POC $\Delta^{14}\text{C}$ values in the North Central Pacific ranged from $+139 \pm 9\%$ at 20 m to $+43 \pm 28\%$ at 5,700 m, and decreased gradually from the surface to the deep sea (Fig. 1a). The value at 20 m is equivalent to the average of sixteen $\Delta^{14}\text{C}$ measurements of DIC ($+132 \pm 6\%$ (1σ)) from 3-m samples taken every other day throughout the 1987 cruise. Similarity of $\Delta^{14}\text{C}$ values in the euphotic zone indicates that suspended POC had been formed recently during photosynthetic fixation of DIC in the surface waters.

$\Delta^{14}\text{C}$ of sinking POC collected in 1987 from the North Central Pacific was $+99 \pm 12$ and $+136 \pm 14\%$ at 4,165 and 5,165 m depth, respectively. The 4,165-m sample had an activity 81% higher than that of suspended POC from the same depth, and the 5,165 m value was higher by 86%, indicating that rapidly sinking, surface-derived particles contribute to the ^{14}C activity of abyssal POC. These elevated ^{14}C activities in the North Central Pacific sinking POC relative to the corresponding suspended POC suggest that there is more reworking within the suspended POC pool than within the sinking pool, so that fewer labile constituents (recently derived from surface waters) remain in the suspended POC pool than in the sinking POC.

$\Delta^{14}\text{C}$ profiles of POC in the Santa Monica Basin are shown in Fig. 1b. Sinking POC $\Delta^{14}\text{C}$ values from three cruises (May 1986, October 1986 and October 1987) display a decrease from an average of $+86 \pm 11\%$ ($N=2$) at 100 m to an average of $+41 \pm 10\%$ ($N=3$) at 850 m. The differences between these profiles are significant and are apparently not solely a function of the seasonal cycle. The $\Delta^{14}\text{C}$ values at 100 m and 200 m for the sinking (78 ± 8 , 94 ± 8 and $101 \pm 8\%$) and suspended ($110 \pm 13\%$) POC at the 900-m station in the Santa Monica Basin are similar to that of the DIC at 5 m ($96 \pm 6\%$), indicating that both POC phases are recent products of photosynthetically produced carbon from surface-water DIC. The sinking POC $\Delta^{14}\text{C}$ values are biased, however, because of appreciable numbers of small

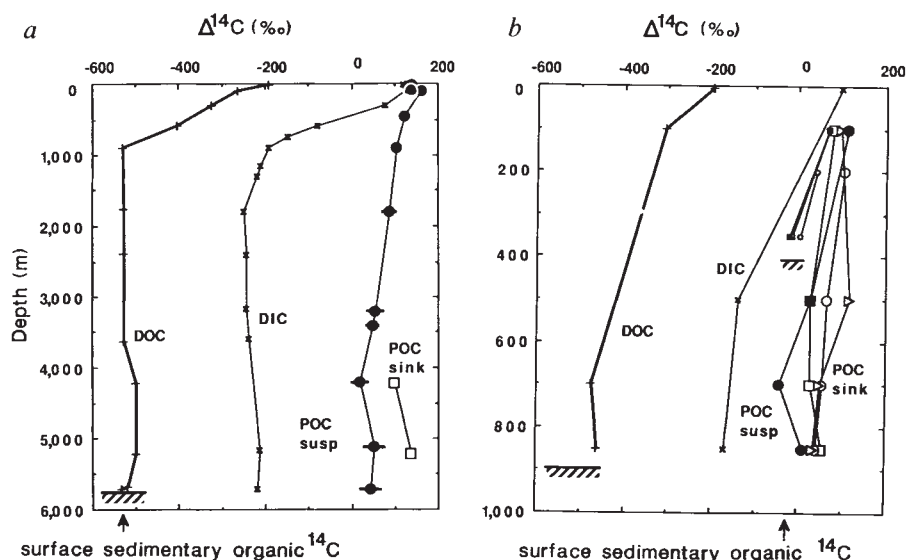
(<1 cm) swimmers in these shallow (100 m, 200 m) trap samples. These swimmers, primarily copepod nauplii, are estimated to account for 10–20% of the total carbon measured.

The $\Delta^{14}\text{C}$ values for suspended POC in the Santa Monica Basin were significantly lower than those for all three sinking POC $\Delta^{14}\text{C}$ profiles, where the largest difference found between sinking and suspended samples was from the same cruise (October 1986; suspended $\Delta^{14}\text{C}$ was 42–120% lower). These lower $\Delta^{14}\text{C}$ values are due at least in part to episodic resuspension of surficial sediment from the shelf^{15,16}, which carries low ^{14}C -activity sediment into the Basin. This is consistent with the lower $\Delta^{14}\text{C}$ values measured for suspended and sinking POC collected at 100 or 200 and 350 m from the eastern shelf of the basin in 400-m-deep water (Fig. 1b). Both the $\Delta^{14}\text{C}$ results from the shelf and other chemical data, including $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ signatures¹⁵, suggest that terrestrial input from the Los Angeles area into the basin waters was minimal, at least during the 1986–87 period. Given the differences in many physical, chemical and biological properties between the NCP and SMB sites, there is a remarkable correspondence in their DOC and suspended and sinking POC ^{14}C activities (Fig. 1), both in profile and in ^{14}C content. Total ultraviolet-oxidizable DOC and suspended POC concentrations below the photic zone are similar at comparable depths at both sites. It is clear that open-ocean ultraviolet-oxidizable DOC is being recycled into the coastal basin and that local perturbations, including higher productivity, do not necessarily dominate deep-basin organic chemistry.

All of the sinking and suspended POC $\Delta^{14}\text{C}$ values in the North Central Pacific and Santa Monica Basin are greater than the corresponding pre-bomb surface DIC values of -50% (ref. 17) and -70% (ref. 18), respectively. This reflects the presence of post-bomb, and thus surface-derived, carbon acquired within the past 30 years throughout the entire water column in both particulate organic phases. If the residence time of suspended POC is the same as that determined for suspended particles⁵ using thorium isotope measurements (5–10 yr), we must assume that suspended POC has turned over at least 2–3 times during the 30-yr lifetime of bomb radiocarbon in the oceans. Furthermore, if all of the POC was surface-derived, we would not expect to see a gradient in $\Delta^{14}\text{C}$ from surface to deep. We believe that,

FIG. 1 a, $\Delta^{14}\text{C}$ of suspended POC, sinking POC, DIC and DOC as a function of depth at $31^\circ 00' \text{N}$, $159^\circ 00' \text{W}$ (North Central Pacific). The value plotted at 1,800 m for the suspended POC is an average of two $\Delta^{14}\text{C}$ analyses ($110 \pm 15\%$ and $62 \pm 15\%$). Symbols on the DOC and DIC profiles are from cruise Eve-1, June–July 1987, with the exception of the four symbols between 1,800 m and 3,600 m on the DIC profile, which are from cruise Alcione-5, October 1985⁹. The shallowest suspended POC value is at 20 m depth. b, $\Delta^{14}\text{C}$ of suspended POC (●, Oct 86), sinking POC (○, Oct 87, ▽, Oct 86, □, May 86), DIC (×) and DOC (+) as a function of depth at $33^\circ 50' \text{N}$, $118^\circ 50' \text{W}$ (Santa Monica Basin). Small open symbols represent $\Delta^{14}\text{C}$ of sinking and suspended POC (as described above) at a 400-m-deep eastern shelf station.

METHODS. $\Delta^{14}\text{C}$ of POC were determined using AMS techniques and are corrected to a $\delta^{13}\text{C}$ of -25% (ref. 14). $\delta^{13}\text{C}$ of POC was measured on CO_2 obtained from the closed-tube combustion step. Errors for the AMS $\Delta^{14}\text{C}$ measurements are ± 5 –8% and those for the conventional $\Delta^{14}\text{C}$ measurements are $\pm 2\%$. $\Delta^{14}\text{C}$ values are corrected for blank CO_2 from the quartz combustion tubes and quartz filters. Blanks composed 1–15% of the sample CO_2 volumes, and had $\Delta^{14}\text{C}$ values of blank CO_2 samples averaging -480% . The error in $\Delta^{14}\text{C}$ for POC is less than or equal to its symbol size except as noted by horizontal bars.



whereas the 5–10 yr residence time does indeed apply to POC, not all components of the suspended (and to a lesser extent the sinking) POC are directly surface-derived in the two areas studied. This would be consistent with the decrease of ^{14}C activities observed in meso-, bathy- and abyssopelagic fishes and crustaceans^{1,2} relative to surface ^{14}C activities.

There are at least three mechanisms that could be responsible for the incorporation of non-surface-derived, low- ^{14}C -activity carbon into the suspended and sinking POC pools. First, low- ^{14}C -activity DOC may be incorporated into the POC pool by heterotrophic uptake of DOC by free-living bacteria in the water column¹⁹. Also, adsorption of DOC onto sub-micrometre webs used by filter feeders such as pteropods and larvaceans, and onto mucus produced in large amounts by various zooplankton (salps, for example) could have a role in sequestering low- ^{14}C -activity DOC (A. Alldredge, personal communication). A mass-balance calculation for the North Central Pacific site requires that only 14% of the deep-sea suspended POC (with an average $\Delta^{14}\text{C}$ of +46‰ between 1,800 m and 5,700 m) need have originated from deep-sea DOC (average $\Delta^{14}\text{C}$ of –520‰ for ultraviolet-oxidizable fraction⁹) to have been responsible for the 93% average reduction of surface POC $\Delta^{14}\text{C}$ values. Fourteen per cent of the deep-sea POC (the mean concentration of which below 1,000 m is $\sim 0.1 \mu\text{mol l}^{-1}$) represents only 0.25% of the deep-sea, ultraviolet-oxidizable DOC ($\sim 40 \mu\text{mol l}^{-1}$)⁹.

Second, chemosynthetic production of organic matter from DIC has been suggested²⁰ to occur at depths of 0–2,000 m in the North Pacific. Deep-sea DIC $\Delta^{14}\text{C}$ values (–240‰ in the North Central Pacific) are also lower than those of POC (Fig. 1a); but a greater portion of the POC (30% or more) would have had to originate by this mechanism to account for the low suspended and sinking POC $\Delta^{14}\text{C}$ values in the deep sea. The amount of POC resulting from *in situ* chemosynthesis can, however, be estimated²⁰ to be only about 5% of the particle flux.

Third, the $\Delta^{14}\text{C}$ value of sedimentary organic carbon (SOC) at the North Central Pacific site (–541‰ in the upper 1 cm of sediment) is comparable to the ^{14}C activities of the ultraviolet-oxidizable DOC in bottom waters. It is difficult to envisage resuspension of SOC as far up into the water column as 1,000 m below the surface at the NCP site, unless it originated from continental-shelf sediments or hydrothermal vent or ridge regions and was advected laterally into the mid-Pacific. In the Santa Monica Basin, however, intermittent resuspension^{15,16} of surficial shelf- and/or slope-derived SOC may contribute to the reduced activity of POC in the water column, especially with respect to the suspended particles. The $\Delta^{14}\text{C}$ activity of this advected material is likely to be similar to that found for surficial SOC at mid-Basin (–10‰, 0–1.25 cm). There is also the possibility of natural oil seeps²² on the basin slopes and anthropogenic petroleum products²³ contributing dead carbon ($\Delta^{14}\text{C}$ = –1,000‰) by adsorption onto particles.

The incorporation of DOC into the POC pool, especially the suspended POC, seems the most reasonable mechanism for lowering POC $\Delta^{14}\text{C}$, as only a small fraction of the ubiquitous low- ^{14}C -activity DOC would suffice to lower the deep-water POC $\Delta^{14}\text{C}$ values, whatever the mechanism. Quantification of the various processes proposed above are complicated by the presence of an extra fraction of DOC²⁴ that was previously undetected by conventional oxidative techniques²⁵. As yet, there are no $\Delta^{14}\text{C}$ measurements for this extra DOC, but it is suspected that the values are different from those found for the ultraviolet-oxidizable fraction because of the correlation between DOC concentration and apparent oxygen utilization at some sites²⁴, and other isotopic evidence²⁶. □

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High-pressure geochemistry of Cr, V and Mn and implications for the origin of the Moon

A. E. Ringwood, T. Kato*, W. Hibberson & N. Ware

Research School of Earth Sciences, Australian National University, Canberra, ACT 2601, Australia

CHROMIUM, vanadium and manganese are present in similar abundances in the Earth's mantle and the Moon, and are substantially depleted relative to their Mg-normalized primordial abundances^{1–6}. Experimental studies^{7,8} of the partitioning of chromium, vanadium and manganese between molten iron and silicates show that these elements are lithophile at the pressures, temperatures and oxygen fugacities prevailing in the Earth's upper mantle and in the Moon. Here, we show that at much higher pressures, corresponding to those in the Earth's lower mantle, the partitioning behaviour of Cr, V and Mn changes owing to increasing solubility of oxygen in molten iron. Cr and V (and perhaps Mn) are preferentially partitioned into molten iron under these conditions. We therefore attribute the depletions of these elements in the Earth's mantle to their siderophile behaviour during formation of the Earth's core, at pressures that were sufficiently high to cause substantial amounts of oxygen to dissolve in molten metallic iron. Similar depletion patterns of Cr, V and Mn in the Earth's mantle and the Moon strongly suggest that a large proportion of the Moon was derived from the Earth's mantle after the Earth's core had segregated.

Chromium, vanadium and manganese are present in near-chondritic abundances (Mg-normalized) in the eucrite parent body^{1–3}. Moreover, Cr and Mn are also present in near-chondritic abundances in the shergottite parent body, believed to be Mars^{4,5}. (The V abundance in the shergottite parent body is poorly constrained.) But these elements are substantially depleted in the Earth's mantle and the Moon^{1–6} and the depletion factors in the Earth's mantle and the Moon are remarkably similar. Seifert and Ringwood⁶ estimated the Cr/Mg ratios of the Earth's mantle and the Moon to be 0.49 and 0.36–0.40 of

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* Present address: Institute of Mineralogy, Petrology and Economic Geology, University of Tohoku, Sendai 980, Japan.

the chondritic value, respectively, and the corresponding V/Mg ratios were estimated to be 0.61 and 0.60–0.72 of the chondritic value. The differences in the abundance patterns between this group of planetary bodies have important implications for their origins.

Recent experimental investigations^{7,8} have shown that Cr, V and Mn behave as lithophile elements during the formation of molten metallic cores in planetesimals and planets ranging in size up to about twice the mass of Mars. Thus, the formation of metallic cores would not cause depletions of Cr, V and Mn in the mantles of these bodies. This is consistent with the evidence that these elements are undepleted in the mantles of the eucrite parent body and Mars^{1–5}.

The chemistry of core formation in the Earth may well have differed from that in Mars and the eucrite parent body because of the much higher pressures and temperatures involved. At the high pressures corresponding to those prevailing in the Earth's lower mantle, FeO would become completely miscible with molten iron^{9,10} and the Earth's core therefore probably contains 8–10 wt% oxygen. Dreibus and Wänke¹ suggested that the presence of this large amount of oxygen in the Earth's core may have influenced the partitioning of Cr, V and Mn, causing them to behave as siderophile elements during the formation of Earth's core, thereby causing their depletions in the mantle. Here we describe the results of experiments designed to test this hypothesis.

Phase relationships in the system Fe–FeO at 16 GPa and 1,600–2,000 °C have recently been determined^{10,11}. There is a eutectic between Fe and FeO at 1,670 °C and the eutectic composition contains 10 ± 2 wt% FeO (equivalent to 2.2 ± 0.5 wt% oxygen). We previously observed¹¹ that at temperatures of 1,670–1,750 °C, for compositions lying on the Fe-rich side of the eutectic, oxygen formerly dissolved in the melt migrates from the liquid to the walls of the alumina capsule during quenching, precipitating as FeO crystals along the boundary. This causes the remaining melt to become oversaturated with metal. Crystals of iron therefore separate, intergrowing to form a framework. Ultimately, pockets of residual metallic liquid near the Fe–FeO eutectic composition become isolated in the framework of interlocking iron crystals, and further diffusion of oxygen to the boundary of the charge is prevented. The trapped pockets of melt crystallize as very fine intergrowths of FeO and metallic iron (Fig. 1). At temperatures between 1,700 °C and the solidus, the composition of this interstitial liquid is very close to that of the eutectic. The pockets of interstitial liquid are mostly ~5 µm in size but may be up to 20 µm across. Under favourable circumstances the composition of the liquid can be determined by

TABLE 1 Partition coefficients between Fe–O melts and crystalline iron

	Runs 454, 458* <i>D</i>	Run 460 (reversal)† <i>D</i>	Combined data <i>D</i>
Cr	5.0 ± 3.3	5.6 ± 1.1	5.2 ± 2.8
V	7.7 ± 6.6	6.6 ± 3.7	7.4 ± 5.8
Mn	3.6 ± 1.7	4.5 ± 1.2	3.9 ± 1.5

Values calculated from measured compositions of pools of former Fe–O metallic liquid and adjoining crystalline iron, as shown in Fig. 1. Run conditions: 16 GPa, 1,700 °C, 30 s.

* Thirteen pairs.

† Six pairs.

electron-probe microanalysis of these areas. We considered that it might be possible to exploit this behaviour to measure the partitioning of Cr, V and Mn between crystalline iron and the Fe–O metallic eutectic liquid from which the iron crystallizes. This would provide a means of evaluating the effects of dissolved oxygen on the partition relationships of Cr, V and Mn between solid and liquid iron.

Experiments were carried out using an MA-8 apparatus operating at 16 GPa, using procedures previously described^{10,11}. We used two starting compositions. The first consisted of an intimate mixture of 93.5 wt% pure iron powder, 5 wt% Fe₂O₃ and 0.5 wt% each of Cr₂O₃, V₂O₅ and MnO. The second mixture comprised 92 wt% of an alloy of Fe₉₉Cr_{0.56}V_{0.39}Mn_{0.13} and 8 wt% Fe₂O₃. The different types of starting materials allowed us to approach equilibrium from different directions. The mixtures were heated slowly (60 min) to 1,630–1,650 °C (below the solidus), and then quickly (a few seconds) to 1,700 °C, just above the solidus, where they were held for 30 s. Runs were quenched by cutting off the power. During the heating cycle, Fe₂O₃ reacted with Fe to form wüstite solid solutions. After recovery the samples were sectioned and studied by optical microscopy and electron-probe microanalysis.

After some preliminary experiments, we decided to use MgO capsules. We found that FeO migrated out of the melt during the run, reacting with the capsule walls to form magnesiowüstite. As before, this left pockets of Fe–O eutectic liquid isolated by interlocking metallic iron crystals, but these pockets were sometimes larger and easier to analyse than those in experiments where Al₂O₃ capsules were used.

The texture of a typical run is shown in Fig. 1. It consists of dispersed pockets of former metallic eutectic liquid scattered throughout a network of iron crystals. The eutectic liquid crystallizes to form a fine feathery intergrowth of FeO + Fe metal,

FIG. 1 Backscattered electron image of polished section of run at 16 GPa, 1,700 °C. During quenching, oxygen migrates towards the boundary of the charge, causing precipitation of crystalline metallic iron (clear, bright regions). Eventually, growth of metallic iron crystals prevents further diffusion of oxygen and the residual, near-eutectic Fe–O melt then crystallizes in the interstices to form fine intergrowths of metallic iron and feathery FeO precipitates (dappled regions).

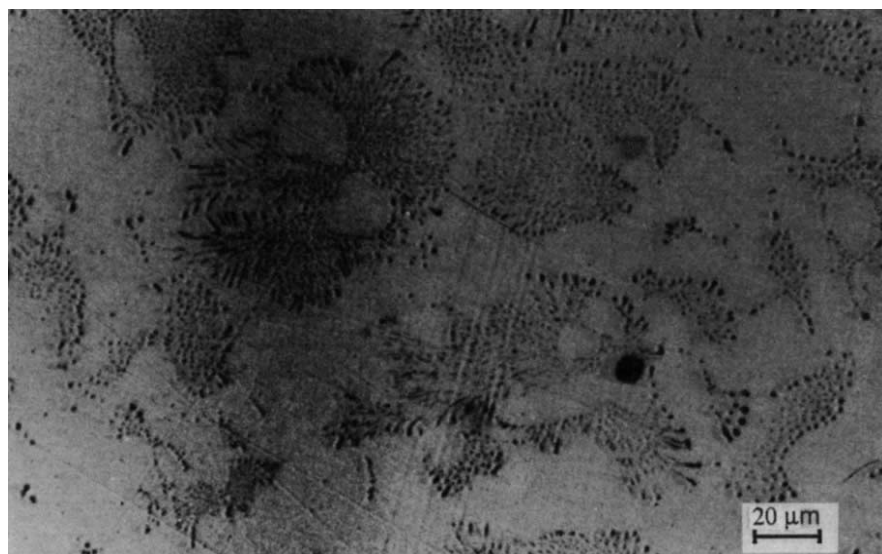


TABLE 2 Experimental and calculated partition coefficients

	D_1^* Perovskite† Cryst Fe	D_2^* Mg wüstite‡ Cryst Fe	$D_3^§$ Pyrolite Cryst Fe	$D_4^¶$ Fe-O melt Cryst Fe	D_5^* Fe-O melt Pyrolite
Cr	3	5	3.4	5.2	1.5
V	4	9	5	7.4	1.5
Mn	8	13	9	3.9	0.4

* Experimental data from ref. 8.

† $\text{Mg}_{0.96}\text{Fe}_{0.04}\text{SiO}_3$ perovskite.

‡ $\text{Mg}_{0.8}\text{Fe}_{0.2}\text{O}$ magnesiowüstite.

§ Calculated from D_1 and D_2 assuming pyrolite comprises 80% perovskite + 20% magnesiowüstite¹³.

|| 80% perovskite + 20% magnesiowüstite.

¶ Experimental data from Table 1.

* Calculated from D_3 and D_4 using the relationship $D_5 = D_4/D_3$.

with most particle sizes being $\leq 1\ \mu\text{m}$. Electron-microprobe analyses were carried out for Fe, O, Cr, V and Mn in pools of former eutectic melt and adjoining iron crystals. The melt regions in 19 of a total of 23 analysed pairs contained an average of 2.2 wt% oxygen (range 1.6–3.2 wt% O), whereas adjoining crystalline iron contained 200–500 p.p.m. oxygen. Partition coefficients D for the distribution of Cr, V and Mn between Fe–O melts and adjacent Fe metal for 19 pairs are given in Table 1. Runs 454 and 458 contained Cr, V and Mn initially as oxides, whereas run 460 used the Fe–Cr–V–Mn alloy. The reversal experiment demonstrates a satisfactory approach to equilibrium. Because of the small sizes of some of the melt inclusions, a significant degree of beam overlap on neighbouring iron crystals probably occurred in some cases. We believe that this is responsible for the anomalously low oxygen contents (0.9–1.4 wt% O) of four melts not included in Table 1.

Our experiments were carried out on compositions close to the Fe–FeO eutectic in which an Fe–O melt is in equilibrium with crystalline iron and wüstite and were therefore at oxygen fugacities close to the iron–wüstite buffer. It is widely believed that the formation of metallic cores in the Earth, the Moon, Mars and the eucrite parent body occurred under redox conditions that were within 1–2 log units of the iron–wüstite oxygen fugacity buffer.

The results demonstrate that the abundances of Cr, V and Mn in the near-eutectic Fe–O melts containing an average of 2.2 wt% oxygen are about 4–7 times higher than in coexisting crystalline iron (Table 1). Studies of the binary systems Fe–Cr, Fe–V and Fe–Mn show that in the absence of oxygen, these elements are concentrated in the melt by factors of only 1.2–1.4^{11,12}. Thus, the presence of only ~2.2 wt% of oxygen in the metallic liquid causes a considerable shift in the partitioning of these elements between molten and crystalline iron such that the concentrations of Cr, V and Mn in the liquid phase are strongly enhanced.

We have previously determined partition coefficients for Cr, V and Mn between Mg-silicate perovskite, magnesiowüstite and crystalline iron at 24.5 GPa and 1,800 °C⁸. The results are given in Table 2. The pyrolite composition of the Earth's lower mantle is approximately equivalent to 80% perovskite + 20% magnesiowüstite¹³. The partition coefficients between a pyrolite lower mantle and crystalline iron, obtained by proportionally weighting these values is given in column 3 of Table 2. Partition coefficients for Cr, V and Mn between an Fe–O melt (~2.2 wt% oxygen) and a pyrolite lower mantle may readily be calculated, as shown in Table 2, using crystalline iron as an internal standard.

Table 2 shows that Cr and V would behave as siderophile elements in the Earth's lower mantle in the presence of molten iron containing only 2.2 wt% of oxygen. It is believed that the amount of oxygen in the Earth's core is much higher than this and may be in the vicinity of 8–10%^{9,10,14}. It seems quite likely

that these higher levels of oxygen would further enhance the siderophile behaviour Cr and V, and it is possible that Mn may also have become siderophile during core formation in the Earth. Thus, it seems reasonable to interpret the depletions of Cr and V in the Earth's mantle, and probably part of the depletion of Mn, to siderophile behaviour during core formation. The fact that Mn is more strongly depleted than Cr and V would not be anticipated from the present data and may reflect some loss of this element as a volatile species³. The depletions of V and Cr cannot be ascribed to volatility-controlled fractionations in the solar nebula, however, because V is less volatile than Mg and Si, whereas Cr possesses a volatility similar to that of Mg and smaller than that of Si under the appropriate cosmochemical conditions^{7,8,13}.

Whereas the present experiments were carried out at a pressure of 16 GPa and at an FeO activity close to unity, the pressures needed to cause significant amounts of oxygen to dissolve in molten iron during formation of the Earth's core would be substantially higher. This is because the activity of FeO in the Earth's mantle is lowered by its solid solution in silicate and oxide minerals, and higher pressures are therefore needed to compensate for this^{9,10}. The solution of oxygen in molten iron during core formation in the Earth in the amounts sufficient to cause siderophile behaviour of Cr and V, would require pressures $>40\ \text{GPa}$ ^{9,10}; such pressures are higher than can be developed in a Mars-sized planetesimal. These considerations, in conjunction with the experimental results described in refs 3, 7 and 8, show that separation of an iron or an Fe–FeS molten core in a Mars-sized planetesimal would not produce depletions of Cr, V and Mn in overlying mantle silicates. This is consistent with the observations that these elements are not depleted in meteorites originating from Mars or from the eucrite parent body. Likewise, the formation of a tiny core in the Moon would not have affected the Cr, V and Mn abundances in the lunar mantle¹³. It therefore seems unlikely that the lunar depletions of these elements were inherited from the mantle of a Mars-sized planetesimal that struck the Earth, as proposed in refs 15 and 16.

The present results, in conjunction with our previous work show that mantle depletions of Cr, V and Mn arising from their siderophile behaviour caused by solution of substantial amounts of oxygen in molten iron, can occur only under the very high pressures reached in the lower mantle of an Earth-sized planet. We conclude, in agreement with Dreibus and Wänke¹, that the depletions of Cr, V and Mn by similar amounts both in the Earth's mantle and Moon are most readily explained if the Moon was formed mainly from material removed from the Earth's mantle after segregation of the Earth's core. A wide range of geochemical evidence based on the behaviour of other siderophile elements in the Earth and the Moon has also been assembled in support of this hypothesis^{5,13,14,17–19}. □

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Neglected taxonomy and continuing extinctions of tuatara (*Sphenodon*)

C. H. Daugherty, A. Cree, J. M. Hay
& M. B. Thompson*

School of Biological Sciences, Victoria University of Wellington,
PO Box 600, Wellington, New Zealand

*Department of Zoology (A08), University of Sydney,
New South Wales 2006, Australia

TAXONOMIC classification is a primary determinant of management priorities for endangered species. Neglect of distinct taxa may lead to their extinction, a problem exemplified by management of the New Zealand tuatara, *Sphenodon*, the only surviving genus of one order of reptiles. The pattern of genetic and morphological differentiation reported here supports a taxonomy dating from 1877 that identified two extant species, one subsequently separated into two subspecies. Tuatara were fully protected in 1895, but legislation and assessments of conservation status never acknowledged taxonomic differentiation, referring only to *Sphenodon punctatus*. Perceived monotypy of tuatara apparently forestalled management intervention on behalf of threatened populations, thus contributing to extinction of 10 of the 40 populations (25%) in the past century and the imminent extinction of four more. Identification of diversity within tuatara warrants increased conservation attention for the single populations of *S. guntheri* (here reinstated as a second living species) and the possibly extinct subspecies *S. p. reischeki*.

The tuatara is almost universally regarded as "a single species... *Sphenodon punctatus*"¹. However, the genus has a complex taxonomic history involving four phases: (1) two extant and one extinct species were named last century (Table 1); (2) in 1904, a catalogue of all New Zealand terrestrial vertebrates listed only *S. punctatus* and included no subspecies²; (3) in 1931 and 1943, variant types of tuatara were formally identified as subspecies of *S. punctatus*^{3,4}, and taxonomic reviews in 1954⁵ and 1977⁶ accepted a single species with three subspecies: *S. p. punctatus*, *S. p. guntheri* and *S. p. reischeki*; (4) studies of tuatara between 1949 and 1981 did not acknowledge any taxonomic differentiation within *Sphenodon*, with one worker observing that "the differences [among proposed taxa] are no greater than have been observed within individual colonies."⁷ This view was generally accepted^{8,9}.

New Zealand legislation protecting tuatara¹⁰ has always referred only to *S. punctatus*, even in 1895¹¹ when a second species, *S. guntheri*, had been recently (1877) described¹². Internationally, the IUCN Red Data Book lists only the taxon *S. punctatus* as 'rare'¹³, despite referring to subspecies for other reptiles, and only *S. punctatus* is included in Appendix I of CITES and in the most recent (1989) taxonomic summary¹⁴. The Red Data Book of New Zealand excludes tuatara entirely,

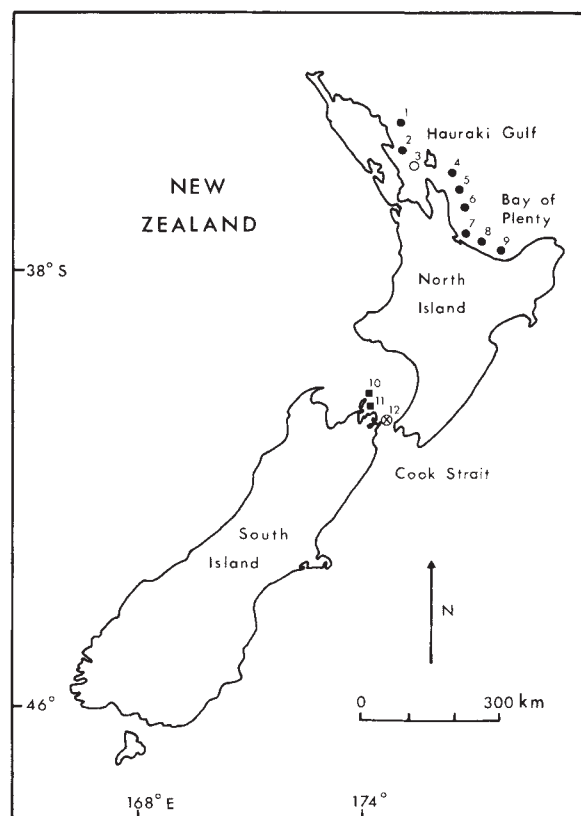


FIG. 1 Distribution of extant, or possibly extant, populations and taxa of tuatara as recognized in this paper. ●, *S. p. punctatus*; ○, *S. p. reischeki*; ■, *S. punctatus*, western Cook Strait type; ⊗, *S. guntheri*. 1, Poor Knights Islands (five islands inhabited by tuatara); 2, Hen and Chickens Islands (four islands); 3, Little Barrier Island; 4, Cuvier Island; 5, Mercury Islands (four islands); 6, Aldermen Islands (seven islands); 7, Karewa Island; 8, Plate Island; 9, Moutoki Island, Rurima group; 10, Stephens Island; 11, Trios Islands (three islands); 12, North Brother Island.

presumably because a single type occurs on ~30 islands (Fig. 1); it claims that "*Sphenodon punctatus*... is neither rare nor endangered."¹⁵ A recent New Zealand listing describes the conservation status of *S. punctatus* as regionally threatened¹⁶. The latter two documents assign conservation status to birds by subspecies, thus implicitly disregarding subspecific differentiation for tuatara.

Because of the discordance of taxonomy, legislation and conservation status, and because no taxonomy has been based on formal analysis of geographic variation, we undertook a survey of allozyme and morphological variation in tuatara. Expeditions in 1988–1989 visited 24 of the 30 islands on which tuatara populations were thought to survive. Six islands were not visited because of the extreme rarity of tuatara or difficulties of access.

TABLE 1 Formally described taxa of tuatara as accepted in this study

Taxon and author	Date	Present distribution	Populations extinct this century
<i>Sphenodon</i> Gray	1831 ²⁵	—	—
<i>S. punctatus</i> (Gray)	1842 ²⁶	—	—
<i>S. p. punctatus</i> (Gray)	1931 ³	24 islands in Hauraki Gulf and Bay of Plenty; plus four islands in Cook Strait that probably warrant separate subspecific recognition	Mokohinau group (3 islands), Motiti I., Slipper I., Shoe I., Whenuakura I., Whale I., Somes I. ^{3,5,15,27}
<i>S. p. reischeki</i> Wettstein	1943 ⁴	Possibly surviving on Little Barrier Island, Hauraki Gulf	None seen on Little Barrier since late 1970s; extinction likely
<i>S. guntheri</i> Buller	1877 ¹²	North Brother Island, Cook Strait	East Island ²⁸
<i>S. diversum</i> Colenso	1886 ²⁹	Extinct; described from bones on East Coast of North Island	—

TABLE 2 Tuatara populations of urgent concern

Taxon	Island (area)	Estimated numbers in population	Confirmed or potential conservation concern(s). Introduced birds
<i>S. p. punctatus</i>	Cuvier (195 ha)	Fewer than 20 adults, no juveniles	Introduced Polynesian rat, <i>Rattus exulans</i> , probably the primary cause of tuatara decline ³⁰ , competes for food (insects), and probably preys on tuatara eggs and hatchlings; introduced cats and goats eradicated. Introduced threatened birds: the insectivorous North Island saddleback, <i>Philesturnus carunculatus rufusater</i> , and the stitchbird, <i>Notiomystis cincta</i>
	Stanley (100 ha)	Fewer than 20 adults, no juveniles; adults apparently not reproducing	Introduced <i>R. exulans</i> ; introduced domestic rabbits eat vegetation, reducing insect populations further. Introduced saddlebacks
	Red Mercury (225 ha)	Fewer than 20 adults, no juveniles	Introduced <i>R. exulans</i> . Introduced saddlebacks; introduced threatened bird, the carnivorous little spotted kiwi, <i>Apteryx owenii</i>
<i>S. p. reischekii</i>	Little Barrier (3,083 ha)	No tuatara seen in 12 yr	Introduced <i>R. exulans</i> ; introduced pigs and dogs eradicated; introduced domestic cats eradicated to protect rare birds. Introduced endangered kakapo or owl parrot, <i>Strigops habroptilus</i> ; introduced threatened kokako, <i>Callaeas cinerea</i> ; introduced saddlebacks, North Island brown kiwi (<i>Apteryx australis mantelli</i>), and great spotted kiwi (<i>Apteryx haastii</i> —did not establish)
<i>S. guntheri</i>	North Brother (4 ha)	Fewer than 300 adults; population reproducing successfully, but limited to 1.7 ha of scrub on top of island	Species limited to one island, for which protection is reduced as result of automation of lighthouse in mid-1990 and departure of resident keepers who have deterred illegal landings and poaching for 123 years

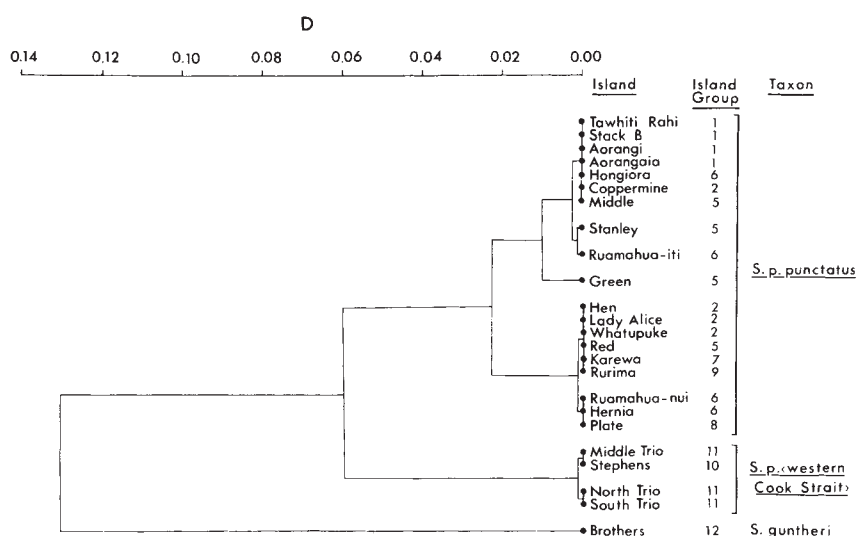
Three distinct groups of tuatara were identified. Most importantly, the population identified as *S. guntheri* in the last century¹² is differentiated from any other population at a minimum of 3 of 25 allozyme loci examined (Fig. 2). Among the other populations (all *S. p. punctatus*), a northern group and a western Cook Strait group can be distinguished. Discriminant function analysis based on seven characters shows the same three groups to be differentiated morphologically, although considerable overlap remains; individuals can be correctly assigned to geographic group with probabilities ranging from 66 to 86% (our unpublished data).

Assignment of precise taxonomic level to divergent congeneric allopatric populations is virtually always subjective¹⁷. In this instance we recommend return of the North Brother Island population to full species status as *S. guntheri* because (1) its relegation to subspecific status was supported by no data³; (2) this is consistent with both the evolutionary and phylogenetic

species concepts¹⁷; and (3) differentiation in allozymes and morphology is equivalent in magnitude to differences between congeneric species pairs of New Zealand lizards whose reproductive isolation is confirmed by sympatry¹⁸, and differentiation in allozymes is equivalent to that commonly found between congeneric species of non-avian vertebrates¹⁹. Western Cook Strait populations are sufficiently distinct from northern populations that we will be assigning them subspecific status within *S. punctatus* in a separate publication. Because conservation programmes should aim to retain the entire range of genetic diversity found in natural populations²⁰, the significance of the data for conservation is in fact independent of the taxonomic labels assigned to the North Brother and the western Cook Straits populations.

S. guntheri was originally assigned species status because of differences 'in appearance', especially 'colour'¹², later supported by differences 'in habits and disposition'²¹. These traits were

FIG. 2 Genetic differentiation and similarities among 24 populations of tuatara, based on weighted pair-group clusterings of Nei's unbiased genetic distance (*D*), calculated from 25 allozyme loci identified in samples of blood taken from a total of 277 individuals. Fifteen loci exhibited no variation; five loci showed only rare alleles expressed in one or several populations; five loci were highly polymorphic. Of the latter, one locus (*Hb-2*) exhibited only local variations within northern populations. The North Brother Island population, *S. guntheri*, was differentiated from all other populations by a fixed difference (apparent apomorphy) at one locus (*Gus-1*) and one nearly fixed difference (*Pgm-1*); a further nearly fixed difference (*Gp-1*) differentiated it from *S. p. punctatus* (Hauraki Gulf, Bay of Plenty); and one nearly fixed difference (*Mdh-1*) differentiated it from *S. punctatus*, western Cook Strait. Island groups are numbered as in Fig. 1. Six populations are not included in this analysis: Little Barrier Island (no animals known to survive); Cuvier Island (few tuatara known to survive); one islet in the Poor Knights group (landing not attempted because of high seas, presence of tuatara not confirmed); and three islets in the Aldermen group (landing not



attempted because of high seas). Only the southern half of Plate Island was sampled.

not included in our analysis; the morphological characters most useful for discriminating *S. guntheri* were length of longest neck spine, snout-vent length, and width of brow ridge. Morphologically, the species of tuatara are cryptic.

Differentiation and taxonomy of *S. p. reischeki* were not assessed, because no surviving tuatara from its only population, Little Barrier Island, is known. However, the taxon *S. p. reischeki* must be considered valid until shown not to be differentiated from other populations of *S. punctatus*.

The tuatara is 'of extraordinary zoological interest'¹³ as the most distinctive surviving reptilian genus in the world, but despite absolute protection of the species and its island habitats, 25% of known populations have become extinct in the past century (Table 1). Management intervention to save any

endangered population has been limited. This contrasts markedly with the highly successful manipulative techniques applied to some threatened New Zealand birds²². Disparities in management intervention, possibly to the detriment of tuatara, are greatest on four islands where tuatara are now on the brink of extinction (Table 2); threatened birds have been introduced and sometimes intensively managed on all four, without intervention on behalf of tuatara.

Taxonomies are not irrelevant abstractions, but the essential foundations of conservation practice²³. The present study shows that neglect of described taxonomic diversity may unwittingly have consigned one subspecies of tuatara, *S. p. reischeki*, to extinction. A second species, *S. guntheri*, has survived on one small island only by good fortune²⁴. □

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Requirement for subplate neurons in the formation of thalamocortical connections

Anirvan Ghosh, Antonella Antonini,
Susan K. McConnell* & Carla J. Shatz

Department of Neurobiology, Stanford University School of Medicine,
Stanford, California 94305, USA

THE neurons of layer 4 in the adult cerebral cortex receive their major ascending inputs from the thalamus. In development, however, thalamic axons arrive at the appropriate cortical area long before their target layer 4 neurons have migrated into the cortical plate. The axons accumulate and wait in the zone below the cortical plate, the subplate, for several weeks before invading the cortical plate. The subplate is a transient zone that contains the first postmitotic neurons of the telencephalon. These neurons mature well before other cortical neurons, and disappear by cell death after the thalamic axons have grown into the overlying cortical plate. The close proximity of growing thalamocortical axons and subplate neurons suggests that they might be involved in interactions important for normal thalamocortical development. Here we show that early in development the deletion of subplate neurons located beneath visual cortex prevents axons from the lateral geniculate nucleus of the thalamus from recognizing and innervating visual cortex, their normal target. In the absence of subplate neurons, lateral geniculate nucleus axons continue to grow in the white matter past visual cortex despite the presence of their target layer 4 neurons. Thus the transient subplate neurons are necessary for appropriate cortical target selection by thalamocortical axons.

The role of subplate neurons in the formation of geniculocortical connections was tested by selectively ablating subplate neurons during development. The neurotoxin kainic acid was injected into the occipital subplate of fetal cats on embryonic day 42 (E42; gestation is 65 days), a time when the first axons from the lateral geniculate nucleus (LGN) have arrived in the visual subplate but before they have entered the cortical plate¹ (see Fig. 1). At this age, many neurons of cortical layer 4 are being generated at the ventricular zone, while others are migrating through the subplate *en route* to the cortical plate. Subplate neurons, by contrast, are generated between E24 and E30 (ref. 2), and have already acquired many characteristics of mature neurons by E42 (refs 3, 4). For example, they (and not other cortical neurons) are sensitive to kainate excitotoxicity; thus a kainic acid injection at E42 selectively kills subplate neurons, leaving the neurons of the cortical plate intact⁵ (see Fig. 2c, d).

During normal development in mammals, LGN axons wait in the subplate below visual cortex from days to months depending on the species^{6–8}. In the cat this occurs between E38 and E50, after which LGN axons first grow into the deep layers of the cortical plate¹ (see Fig. 1; A.G. and C.J.S., manuscript in preparation). Geniculocortical axons can be visualized by placing crystals of DiI into the LGN of an aldehyde-fixed brain (Fig. 2). Figure 2a shows this projection in an E60 control brain injected with saline at E42. As in normal animals, LGN axons leave the optic radiations to branch extensively in the subplate and in the deep layers of the visual cortex. (Deep-layer neurons which project subcortically are also labelled retrogradely, as expected⁹.) To determine how the absence of subplate neurons affects LGN axons, we examined the morphology of these axons at E60, after kainic acid had been injected to delete subplate neurons at E42. In these animals, the entire zone that normally contains subplate neurons and waiting axons has collapsed, and LGN axons no longer arborize below the cortical plate (Fig. 2b). Instead of fanning out and branching in the subplate, the axons grow past the visual cortex in a tight fascicle. These

* Present address: Department of Biological Sciences, Stanford, California 94305, USA.

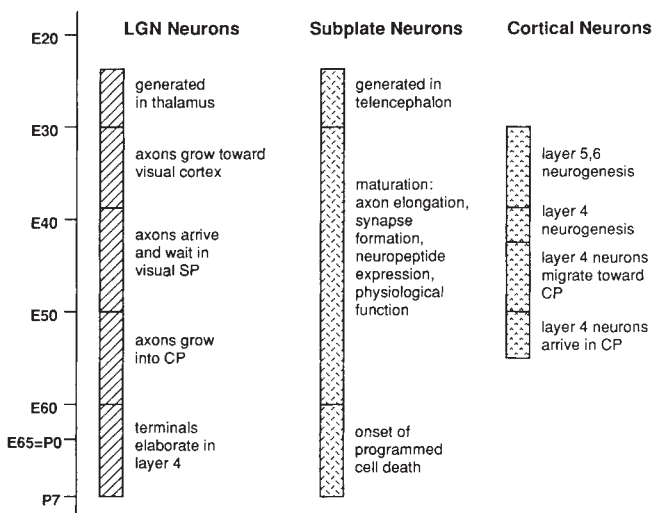


FIG. 1 Timing of events associated with the development of the thalamocortical pathway, subplate neurons, and layer 4 cortical neurons, in the visual system of the fetal cat. Abbreviations: SP, subplate; CP, cortical plate (for review, see ref. 8).

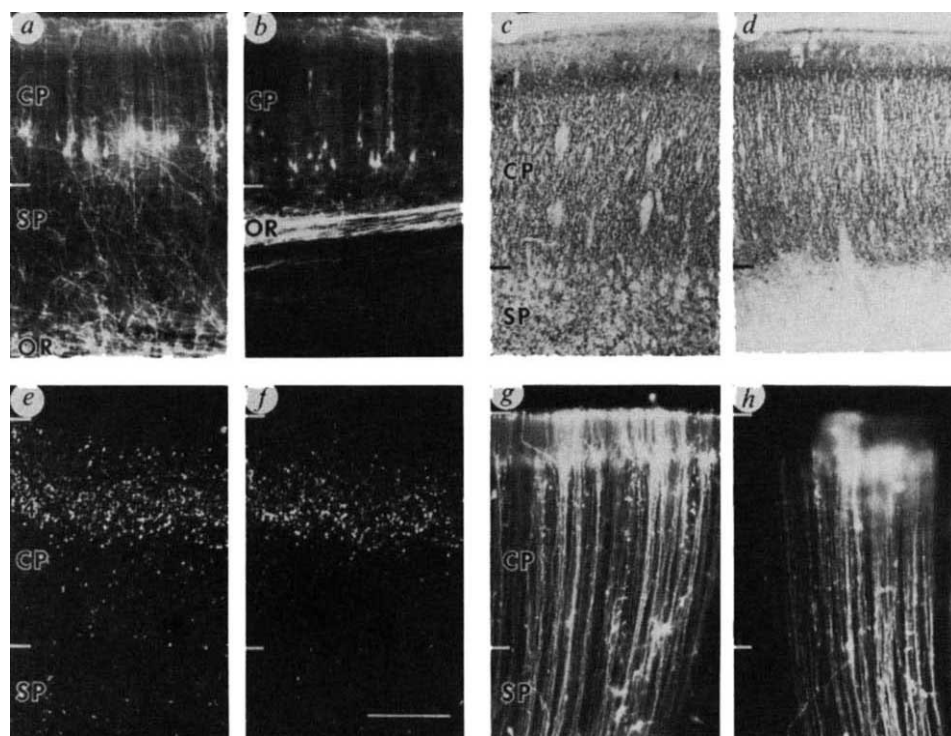
observations suggest that subplate neurons are necessary for LGN axons to stop and arborize below their normal cortical target before they grow into the cortical plate.

Several control experiments were performed to determine whether these effects are specifically due to the absence of subplate neurons. First, MAP2 immunocytochemistry was used to examine the histological organization of the cortical plate and subplate after injections of kainic acid or saline at E42. Both subplate neurons and cortical plate neurons stain heavily for MAP2 at E60 in saline-injected controls (Fig. 2c). By contrast, in brains lesioned by kainic acid, immunoreactivity is completely absent from the subplate (Fig. 2d), as has been shown previously⁵. The intensity of staining and the thickness of the cortical plate are unaffected by the lesion, consistent with the suggestion that cortical plate neurons are too immature at E42 to be susceptible to kainate toxicity.

The effect of kainic acid lesions at E42 was also examined on two other cell types that might be required for the normal development of LGN axons: radial glial cells, the predominant glial subtype in the developing cortex^{10,11}, and layer 4 neurons, the ultimate targets of thalamic axons. The morphology and patterning of radial glial cells revealed by DiI labelling were indistinguishable in the saline- and kainic acid-injected hemispheres at E60 (Fig. 2g, h), indicating that this cell type is not destroyed by injections of kainic acid at E42. Second, to confirm that the genesis, migration and survival of layer 4 neurons were not affected, we labelled these cells with [³H]thymidine

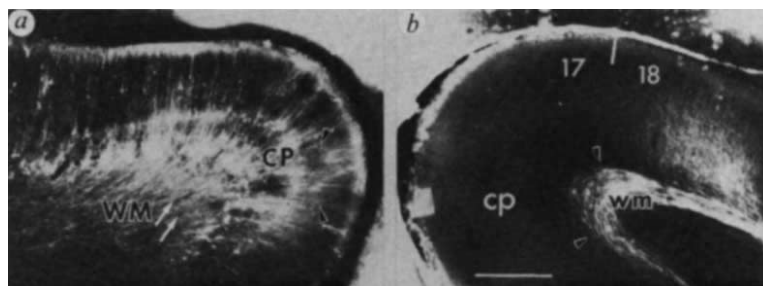
FIG. 2 Sections through the visual cortex at E60 after intracortical injections of kainic acid (b, d, f, h) or saline control (a, c, e, g) at E42, showing that kainic acid lesions result in the specific ablation of subplate neurons and marked changes in LGN axon morphology at E60. a, b, In the control hemisphere (a), anterogradely labelled LGN axons leave the optic radiations (OR) to branch below the visual cortex in the subplate (SP). Retrogradely labelled neurons in the deep cortical layers (CP) are also present as expected, as they project subcortically. In the kainate-lesioned hemisphere (b), LGN axons fail to branch and remain fasciculated in the OR, running past the visual cortex in a tight bundle. c, d, Extent of ablation verified by MAP2 immunocytochemistry⁵. c, Control hemisphere shows dense MAP2 immunoreactivity associated with the somata and the dendrites of neurons in both the cortical plate and subplate. d, In the kainate-lesioned hemisphere, cortical plate neurons remain and exhibit MAP2 immunoreactivity, but the subplate neurons are missing. The thickness of the cortical plate is indistinguishable from that of controls. e, f, Dark-field autoradiographs of the visual cortex on E60, after injection of [³H]thymidine on E42 to label layer 4 neurons¹². Layer 4 neurons have migrated into their normal positions in both the control (e) and kainic acid-lesioned (f) hemispheres. g, h, DiI-labelled radial glial cells (see methods) are indistinguishable in control (g) and kainate-treated (h) hemispheres. Scale bar (shown in f): 450 μ m (a, b); 300 μ m (c-h).

METHODS. Subplate neurons were ablated by injecting 0.6–0.8 μ l of 10 mg ml⁻¹ kainic acid (Sigma) in 0.9% NaCl into the visual subplate in one hemisphere in fetal cats at E42 as previously described⁵. The other hemisphere was injected with an equal volume of 0.9% NaCl (saline) as a control. Fetal surgeries were performed under halothane anaesthesia using sterile surgical procedures². In four fetuses, 500 μ Ci [³H]thymidine in 0.1 ml sterile saline was injected intraamniotically after the kainate injections to label



layer 4 neurons. A total of 11 fetuses were injected with kainic acid at E42 and perfused on E50(2), E59(1), E60(2), E63(4), P5(1) or P8(1) with 4% paraformaldehyde. To visualize the geniculocortical pathway, small crystals of DiI (Molecular Probes, D282) were placed into the LGN of fixed brains. The brains were stored in the same fixative for 2–4 months to allow for the diffusion of the dye^{14,17}. Coronal sections (200 μ m) were cut on a vibratome and viewed under rhodamine epifluorescence optics. Radial glial cells were visualized by preparing 1-mm thick coronal slices of aldehyde-fixed brains, into which crystals of DiI were placed into the ventricular zone to label radial glial processes by means of their ventricular endfeet. MAP2 immunocytochemistry and [³H]thymidine autoradiography were performed as described previously^{2,5}.

FIG. 3 LGN axons examined in postnatal life have failed to recognize and innervate visual cortex after subplate ablation at E42. *a*, Coronal section through the visual cortex of a normal animal at P2 shows a wealth of labelled LGN axons in the cortical plate, as well as retrogradely labelled pyramidal neurons in the deep cortical layers, resulting from a Dil injection into the LGN. LGN axons fan out radially as they enter the cortex (arrows) and branch extensively in layer 4 (arrowheads). *b*, Section through the visual cortex at P5 after ablation of subplate neurons underlying primary visual cortex (area 17) at E42 shows that LGN axons fail to recognize and innervate primary visual cortex and instead grow past the area, confined to the white matter (wm, arrowheads). These axons can be followed underneath the splenic sulcus and into the cingulate gyrus (not shown). A subset of LGN axons have entered area 18, a normal target of LGN axons where subplate neurons are spared. Scale bar, 750 μ m.



METHODS. Subplate neurons were ablated with kainic acid, and geniculocortical axons were labelled with Dil as described in Fig. 2.

immediately after injection of kainic acid and followed their subsequent development. Previous [3 H]thymidine-labelling studies showed that neurons comprising the upper half of layer 4 are born at this time¹². Autoradiographs of control and kainic acid-treated brains at E60 (Fig. 2*e,f*) indicate that layer 4 neurons generated on E42 can indeed migrate through the kainic-treated zone and take up their appropriate position in the cortical plate.

Finally, to address the possibility that LGN axons might be directly affected by the kainate treatment, we examined whether axons exposed early on to kainic acid would still innervate the cortical plate later on. Kainic acid was injected into the subplate below temporal cortex (future auditory cortex) at E38, just when LGN axons *en route* to visual cortex are growing past. Subsequent Dil injections into visual cortex at E50 retrogradely labelled LGN cells, implying that exposure to kainate had not compromised the ability of these LGN axons to innervate cortex (A.G. and C.J.S., manuscript in preparation). Thus it seems unlikely that LGN axons are affected directly by the kainic acid injection. Taken together, these controls strongly suggest that the effects of the kainic acid lesion on LGN axons are specifically due to the absence of subplate neurons.

To determine whether LGN axons can eventually recognize and innervate the visual cortex despite the absence of the subplate, we deleted subplate neurons at E42 and labelled LGN axons with Dil 30 days later, at postnatal day (P) 5. In normal animals, LGN axons grow into layer 4 during the first postnatal week^{1,13}. Figure 3*a* shows that at P2 both anterogradely labelled axons and retrogradely labelled neurons in the deep cortical layers were present. As expected at this age, LGN axons fan out radially as they enter the cortex and branch extensively in layer 4.

A section through visual cortex at P5 following an E42 subplate deletion is shown in Fig. 3*b*. In contrast to that of normal animals, the trajectory of LGN axons is radically modified after a deletion of subplate neurons. LGN axons fail to innervate the primary visual cortex, their appropriate target. Instead, these Dil-labelled axons have grown past their normal cortical target in a dense fascicle restricted to the white matter, seemingly unaware of their correct cortical destination. Serial sections spanning the extent of visual cortex show that where subplate neurons are missing, the entire geniculocortical projection has been rerouted. The axons grow past visual cortex and into the white matter underlying the adjacent cingulate gyrus (not shown); we do not so far know whether these axons invade and innervate this inappropriate cortical area.

A subset of LGN axons does, however, enter area 18, a normal target of LGN axons (Fig. 3*b*). MAP2 immunocytochemistry and cresyl violet staining show that although subplate neurons are absent underneath area 17, they are present below area 18 in the region where axons have entered the cortical plate. Thus, where subplate neurons are present, LGN axons can innervate

directly overlying cortical areas. Subplate neurons may also have a role in the formation of the feedback connection from the cortex. We have previously shown that subplate neurons pioneer the first axon pathway from cortex to thalamus very early in development, a pathway that is followed later by corticofugal axons¹⁴. Consistent with this observation, few cortical layer 6 neurons could be retrogradely labelled from the LGN after subplate ablation (Fig. 3*b*), suggesting that these cortical neurons also fail to innervate their appropriate thalamic target in the absence of subplate neurons.

The results of this study imply that interactions between growing geniculocortical axons and subplate neurons are required for axons to select and grow into their appropriate target, the visual cortex. Thus, one function of subplate neurons may be to mediate the formation of orderly sets of connections between different thalamic nuclei and their appropriate cortical target areas.

Much discussion has addressed the issue of how cortical areas are specified during development^{15,16}. One view is that cortical areas have intrinsic differences that are mapped out very early in development, perhaps even in the ventricular zone. Alternatively, cortical areas may emerge gradually from an undifferentiated 'protocortex' through epigenetic influences such as afferent inputs. Our results indicate that subplate neurons stand as a crucial link in cortical target recognition, whichever strategy is used. The observation that LGN axons grow past the visual cortex in the absence of subplate neurons indicates that the cortical plate alone has insufficient information to allow the ingrowth of appropriate axons. It remains to be seen whether in these animals, thalamic axons invade and innervate layer 4 of inappropriate cortical regions as a result of subplate ablation. Such findings could shed light on the issue of whether intrinsic differences among subplate neurons specify the identity of overlying cortical areas. □

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Arachidonic acid released from striatal neurons by joint stimulation of ionotropic and metabotropic quisqualate receptors

Aline Dumuis, Jean Philippe Pin, Kiyoshi Oomagari, Michèle Sebben & Joël Bockaert*

Centre CNRS-INSERM de Pharmacologie-Endocrinologie,
Rue de la Cardonille, 34094 Montpellier Cedex 5, France

ASSOCIATIVE stimulation of *N*-methyl-D-aspartate (NMDA) receptors and quisqualate ionotropic receptors (Q_i) induces long-term potentiation at particular glutamatergic synapses¹⁻⁷. Release of arachidonic acid as a result of stimulation of NMDA receptors⁸ has been proposed to play a part in the establishment of long-term potentiation⁹⁻¹¹. But long-term plasticity events at some other glutamatergic synapses do not involve activation of NMDA receptors¹¹⁻¹⁵. Here we report that in mature striatal neurons in primary cultures, quisqualate can release arachidonic acid by associatively activating both quisqualate metabotropic receptors coupled to phospholipase C (Q_p) (refs 16-20) and Q_i receptors²¹. Independent activation of these two receptor types with specific agonists did not stimulate arachidonic acid release. These results support a role for the associative activation of Q_p and Q_i receptors in synaptic plasticity events¹²⁻¹⁴, including long-term potentiation at particular synapses^{11,15}.

To study the effect of excitatory amino acids on the release of arachidonic acid (AA), cultures of striatal neurons were prepared as previously described²², but maintained for 13 days *in vitro*. These neuronal cultures do not contain more than 7% of glial cells, as revealed by counting cells positive for glial fibrillary acidic protein^{22,23}. Unless otherwise indicated, experiments were performed in the absence of Mg^{2+} to avoid NMDA-associated channel blockage²⁴. After a 24-h preincubation with [³H]AA, neurons released [³H]AA on stimulation with various excitatory amino acids. The efficacy of glutamate in releasing [³H]AA ($620 \pm 78\%$ increase over the basal release, $n = 10$) was more than twice that of NMDA ($245 \pm 49\%$, $n = 7$) or that of quisqualate (QA; $210 \pm 35\%$, $n = 6$) (Fig. 1). The potencies of QA, NMDA and Glu were $1.25 \pm 0.16 \mu M$ ($n = 10$), $30 \pm 7 \mu M$ ($n = 7$) and $10.3 \pm 2.4 \mu M$ ($n = 6$), respectively (Fig. 1). The QA-induced [³H]AA release was not mediated by NMDA receptors as it was not sensitive to either a competitive antagonist (2-amino-5-phosphonovaleate; $100 \mu M$) or to several noncompetitive antagonists ($10 \mu M$ phencyclidine, $1 \mu M$ MK-801, or $2 mM$ Mg^{2+}) of NMDA receptors^{24,25} (not shown). Moreover, QA ($100 \mu M$) and NMDA ($100 \mu M$) induced [³H]AA release ($212 \pm 66\%$ and $269 \pm 40\%$ increase over basal release, respectively ($n = 4$)) that was additive ($458 \pm 92\%$, $n = 4$). In addition, the Glu response measured in the presence of $1 \mu M$ MK-801 ($197 \pm 43\%$, $n = 3$) was similar to the QA effect. These results indicate that in the absence of Mg^{2+} , AA release from neurons maintained for 13 days *in vitro* and stimulated by Glu resulted from the activation of both NMDA and QA receptor types.

The nature of the [³H]AA metabolites released by these different excitatory amino acids has been examined by HPLC separations of chloroform extracts of the medium. After Glu, QA or NMDA stimulation, the radioactivity released from the cells was exclusively eluted as AA (not shown). No trace of hydroxyeicosatetraenoic acids or prostaglandins could be detected.

The nature of the QA receptors involved in [³H]AA release was then analysed. A selective antagonist of the Q_i receptor²⁵ that does not block the Q_p receptor²⁶ 6-cyano-2,3-dihydroxy-7-nitro-quinoline (CNQX), totally inhibited the QA-induced [³H]AA release in a dose-dependent manner (Fig. 2a). The CNQX affinity in blocking this QA response (inhibition constant, $K_i = 3.66 \pm 0.55 \mu M$, $n = 6$) was similar to that reported on many other Q_i -mediated responses²⁷ (K_i was calculated according to Cheng and Prusoff equation²⁸). This indicates that the activation of Q_i receptors is necessary for the QA-induced release of [³H]AA release. This QA response also has characteristics of a Q_p -mediated response such as its inhibition by phorbol-12,13-dibutyrate (PdBu) (Fig. 2b), which blocks most of the receptors associated with phospholipase C, including the Q_p receptors in striatal neurons²⁹. The specificity of this PdBu effect is indicated by two observations: (1) 4α -phorbol 12,13-didecanoate (PdDc), an inactive analogue of PdBu, did not reduce the QA response (Fig. 2b); and (2) NMDA-induced AA release in the absence of Mg^{2+} was not reduced by PdBu. NMDA ($100 \mu M$) stimulated by $267 \pm 33\%$ ($n = 3$) and $290 \pm 36\%$ ($n = 3$) over basal AA release in the absence and presence of PdBu ($0.1 \mu M$), respectively. We demonstrated, by measuring the increase in intracellular Ca^{2+} (with the fura-2 technique; O. Manzoni *et al.*, manuscript in preparation) or the stimulation of GABA (γ -aminobutyric acid) release³⁰ that PdBu did not affect the Q_i -mediated responses (data not shown).

These results led us to focus our attention on the possible cooperation between Q_i and Q_p receptors to stimulate [³H]AA release. The agonist α -amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid (AMPA), a potent and selective Q_i agonist^{18,19,21,30} elicited only a weak effect in stimulating [³H]AA

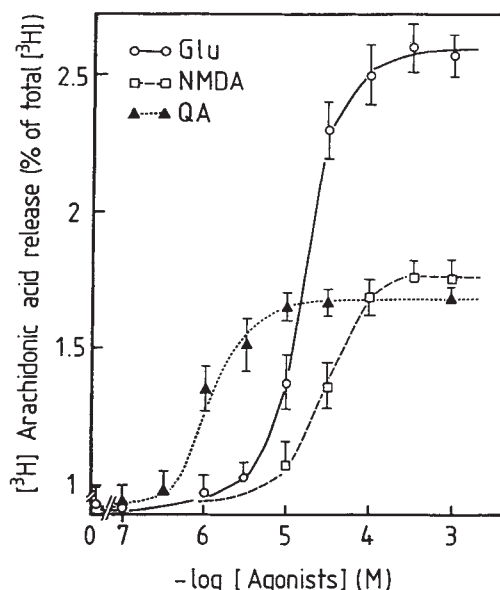


FIG. 1 Glu, NMDA and QA dose-dependently stimulate [³H]AA release from striatal neurons maintained for 13 days *in vitro*. The percentage of radioactivity released from the cells during a 15-min stimulation with increasing concentrations of Glu (○), NMDA (□) or QA (▲) is presented. Results are means $\pm$ s.e.m. of six separate experiments performed in triplicate. **METHODS.** Striatal neurons maintained for 13 days *in vitro* were incubated overnight in six-well Costar dishes with 0.25 or $5 \mu Ci ml^{-1}$ [³H]AA ($166 Ci mmole^{-1}$, CEA, Saclay France). Cells were washed five times with $2 ml$ per well of Krebs bicarbonate buffer ($3.5 mM$ KCl, $0.75 mM$ $CaCl_2$, $124 mM$ NaCl, $1.25 mM$ K_2HPO_4 and $26.3 mM$ $NaHCO_3$, pH 7.4) containing 0.05% BSA free of fatty acids and equilibrated with $95\% O_2/5\% CO_2$. Neurons were stimulated for 15 min with the indicated concentrations of Glu, NMDA or QA. The radioactivity released in the medium ($[^3H]med$) and that remaining in the cells ($[^3H]cell$) was determined. The percentage of radioactivity released from the cells was calculated as $100 \times [^3H]med / ([^3H]med + [^3H]cell)$.

* To whom correspondence should be addressed.

release (Fig. 3). *Trans*-1-amino-cyclopentyl-1,3-dicarboxylic acid (ACPD) is a selective agonist of Q_p receptors^{21,31,32} and can stimulate inositol phosphate production when applied alone on striatal neurons³². In the same model, ACPD alone had a very weak effect on [3 H]AA release (Fig. 3). These results clearly indicate that the specific activation of Q_i receptor with AMPA or of Q_p receptors with ACPD is not sufficient to stimulate AA release.

By contrast, ACPD has a strong effect in the presence of 30 μ M AMPA (Fig. 3). The efficacy of ACPD in the presence of AMPA was similar to that of QA and its potency in inducing [3 H]AA release was similar to its potency in stimulating inositol phosphate production in the same system³² (50% effective concentration, $EC_{50} = 45 \pm 18$ μ M, $n = 4$). As expected, the response induced by ACPD (300 μ M) plus AMPA (30 μ M) was inhibited by PdBu (50 nM) treatment of the cells ($72 \pm 8\%$, $n = 3$, inhibition). Clearly, the QA-induced [3 H]AA release resulted from the associative stimulation of both Q_i (AMPA-sensitive) and Q_p (ACPD-sensitive) receptors. The parallel between this associative property of Q_i and Q_p receptors and the associative property of Q_i and NMDA receptors to stimulate [3 H]AA release was striking. Indeed, in the presence of physiological concentrations of Mg^{2+} , we could demonstrate that NMDA (100 μ M) stimulates [3 H]AA release only in the presence of AMPA (30 μ M) (Fig. 3 insert).

The associative stimulation of Q_i and Q_p receptors in releasing [3 H]AA is probably mediated through an activation of phospholipase A2, as this response was inhibited by mepacrine, an inhibitor of phospholipase A2 ($31.2 \pm 12.0\%$ of control at 10 μ M), but not by RHC80267, a selective inhibitor of diacylglycerol lipase³³ ($109 \pm 7\%$ of control at 10 μ M).

Association between two events—that is, presynaptic stimulation and postsynaptic depolarization—in the central nervous system is always important as it is central to models of associative learning, based on the Hebb hypothesis³⁴. As NMDA receptor channels are blocked by Mg^{2+} at normal membrane potential, the opening of NMDA channels at glutamatergic synapses also requires presynaptic stimulation to release the agonist glutamate and postsynaptic depolarization to remove the Mg^{2+} blockade. In some cases (of homosynaptic long-term potentiation) the postsynaptic depolarization may be due to the activation of

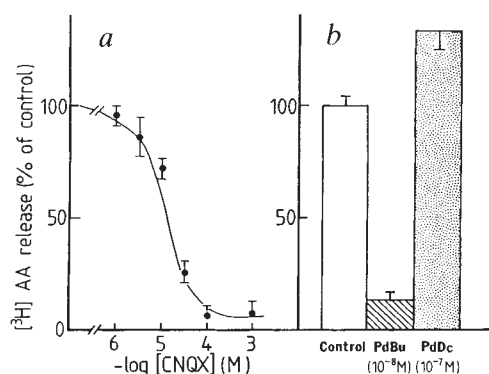


FIG. 2 The QA-induced AA release exhibits properties characteristic of both Q_i and Q_p receptors. *a*, Inhibition by CNQX of QA-induced [3 H]AA release. *b*, Effects of PdBu and PdDc on the QA-stimulated [3 H]AA release. Results are expressed as the percentage of maximal QA response. Maximal QA-induced [3 H]AA release minus base was $1.12 \pm 0.24\%$ of radioactivity released from the cells. Results are means $\pm$ s.e.m. of four separate experiments performed in triplicate.

METHODS. Striatal neurons maintained for 13 days *in vitro* were exposed to 10 μ M QA for 15 min in the presence of increasing concentrations of CNQX (*a*). *b*, Control neurons (without treatment) or neurons first exposed either to 50 nM PdBu or to 100 nM PdDc for 10 min before starting experiments, were then exposed to 10 μ M QA for 15 min. The percentage of [3 H]AA released from the cells was determined as in the legend to Fig. 1.

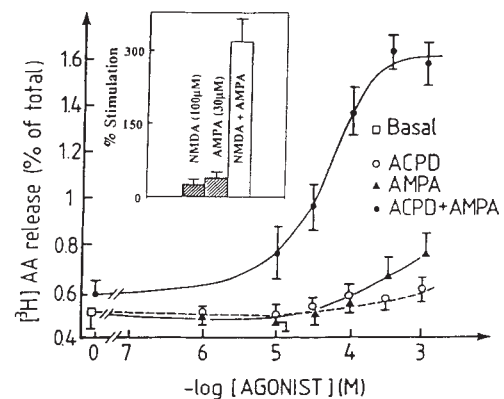


FIG. 3 Simultaneous activation of Q_p and Q_i receptors is needed to stimulate [3 H]AA release. Effects of increasing concentrations of AMPA ($\blacktriangle$), ACPD alone ($\circ$) or ACPD + 30 μ M AMPA ($\bullet$) on [3 H]AA release from striatal neurons. Results are expressed as the percentage of radioactivity released from the cells. Inset: The effects of AMPA (30 μ M), NMDA (100 μ M) or NMDA (100 μ M) and AMPA (30 μ M) in the presence of 2 mM Mg^{2+} are presented. Results in inset are expressed as the percentage of stimulation over basal release ($0.48 \pm 0.11\%$ of radioactivity released from the cells). Results are means $\pm$ s.e.m. of at least four independent experiments performed in triplicate. Methods as described in the legend to Fig. 1.

another glutamatergic receptor, the Q_i receptor, located in the same synapse.

We have demonstrated here that the release of AA by QA also necessitates the association between activation of a specific Glu receptor type (the Q_p one) and depolarization triggered by Q_i receptor activation. Preliminary results indicate that other depolarizing agents, such as low concentrations of veratridine, also allowed activation of Q_p receptors to stimulate AA release.

We point out that the associative activation of either Q_i and NMDA receptors, or Q_i and Q_p receptors produce qualitatively the same pattern of signals: depolarization, Na^+ entry, increase in intracellular Ca^{2+} , protein kinase C activation and AA release^{5,8,29}. It has been proposed that AA could play an important part in synaptic plasticity, at least that involving activation of NMDA receptors. Williams and co-workers showed that AA may have a role in the genesis of long-term potentiation¹⁰ and several groups have reported a blockade of long-term potentiation induction with inhibitors of phospholipase A2 (refs 7, 11). As the association of non-NMDA receptor activation and postsynaptic excitation also induces synaptic plasticity at least in the CA3 area of the hippocampus^{5,11,15} and in the cerebellum¹²⁻¹⁴, our results showing that neuronal excitation associated with activation of Q_p receptors is required for AA release, further support the involvement of Q_i - Q_p receptor association in plasticity events. □

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Primary structure and functional expression of a cyclic nucleotide-activated channel from olfactory neurons

Ravinder S. Dhallan*, King-Wai Yau†, Karen A. Schrader‡ & Randall R. Reed†‡§

* Department of Biomedical Engineering, † Department of Neuroscience, and ‡ Department of Molecular Biology and Genetics, The Howard Hughes Medical Institute, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205, USA

ODORANT signal transduction occurs in the specialized cilia of the olfactory sensory neurons. Considerable biochemical evidence now indicates that this process could be mediated by a G protein-coupled cascade using cyclic AMP as an intracellular second messenger¹. A stimulatory G protein α subunit is expressed at high levels in olfactory neurons and is specifically enriched in the cilia², as is a novel form of adenylyl cyclase³. This implies that the olfactory transduction cascade might involve unique molecular components. Electrophysiological studies have identified a cyclic nucleotide-activated ion channel in olfactory cilia⁴. These observations provide evidence for a model in which odorants increase intracellular cAMP concentration, which in turn activates this channel and depolarizes the sensory neuron. An analogous cascade regulating a cGMP-gated channel mediates visual transduction in photoreceptor cells (see refs 5, 6 for review). The formal similarities between olfactory and visual transduction suggest that the two systems might use homologous channels. Here we report the molecular cloning, functional expression and characterization of a channel that is likely to mediate olfactory transduction.

The coding region of the cGMP-gated channel from bovine rods⁷ was isolated and used to screen a rat olfactory complementary DNA library at low stringency (see Fig. 1a methods). A single class of recombinant clones was identified. Sequence analysis of the longest of these cDNA clones (clone 3) revealed an open reading frame of 664 amino acids (Fig. 1a). The putative protein encoded by this cDNA clone shares considerable homology with the photoreceptor channel (57% identity). One region of sequence divergence occurs at the amino terminus of the two proteins, and is predicted to reside on the cytoplasmic side of the membrane⁷. The remainder of the coding region of these proteins exhibit extensive homology. Notably, the region near the carboxyl terminus implicated as the cyclic nucleotide binding site⁷ (overlined region in Fig. 1a) is highly conserved. The hydropathicity profile of the rod channel suggests perhaps four or six membrane-spanning regions⁷. The nearly identical

hydropathic profiles (Fig. 1b) and the high level of overall homology shared by the rod and the olfactory channels do not allow further resolution of possible models for the topological distribution of the membrane-spanning segments proposed previously⁷. In any case, the two channel proteins are likely to have the same topology.

A second full-length independent cDNA clone (clone 2) was also sequenced. The two clones (2 and 3) differ by three nucleotides in length at the 5' end, the length of the poly(A) tail (13 versus 9 nucleotides) and a single internal position (G versus A at nucleotide 635) that results in a lysine-arginine change in the protein sequence. A third independent cDNA clone contains the A residue, as does clone 3, at this position. The difference could have arisen during cDNA synthesis or as a natural variant in the animal population.

The tissue distribution and abundance of the messenger RNA encoding this putative olfactory channel was determined by northern blot analysis (Fig. 2a, right panel). An abundant 3.2-kilobase (kb) message is seen only in RNA from olfactory epithelium. The expression of this message is reduced on elimination of the sensory neurons induced by olfactory bulbectomy⁸ (Fig. 2a, left panel). These results suggest the channel is expressed in olfactory neurons. A similar pattern of expression is seen for the G protein α -subunit (G_{olfa}) specific to olfactory neurons (ref. 2).

The extensive homology shared by the olfactory and the visual channels isolated from different species raised the possibility that they might be derived from the same gene. However, Southern blot analysis of genomic DNA from several species (Fig. 2b) revealed unique patterns of hybridization when the coding region of the two channels were used as probes. This observation indicates that the two channels are the products of distinct genes and that they do not have any genomic sequences in common.

The electrophysiological properties of the olfactory channel were examined by transient expression in a human embryonic kidney cell system^{9,10}. Inside-out patches of plasma membrane¹¹ were excised from the transfected cells and tested for sensitivity to bath-applied cyclic nucleotides. At 2 days after transfection, 22 out of 53 patches showed a cyclic-nucleotide-induced current. At earlier times, the frequency of responsive patches was lower, as was the amplitude of the membrane current. In control experiments a total of 50 patches were excised from mock-transfected cells; none of these responded to cyclic nucleotides.

FIG. 1 a, The DNA and deduced amino-acid sequences (single-letter code) of the rat olfactory channel. The initiating methionine has been assigned position +1. The tandem ATG codons that initiate the open reading frame are preceded by stop codons in all three frames. The second of these methionine residues is in better context for translation initiation²⁰. The third row of each line displays the protein sequence of the bovine rod channel⁷; only differences are indicated, and no attempt was made to align the first 150 amino acids of the rod channel. No gaps are required to align the sequences of the two channels, with the exception of a single position at bovine Lys 619, which has no counterpart in the rat olfactory channel; this has been inserted in the displayed sequence as K. The region proposed to bind cyclic nucleotides is overlined. **b**, Hydropathicity comparison of the olfactory and visual channels by the method of Kyte and Doolittle²¹. Hydrophobic regions are shown with positive values.

METHODS. The bovine rod channel probe was obtained using the polymerase chain reaction with oligonucleotides corresponding to the sequences at positions -49–-27 and 2,139–2,166 (ref. 7). The 5' oligonucleotide contained an *EcoRI* site at the 5' end. The oligonucleotides were used to prime synthesis from 1 μ l of an amplified bovine retinal library (5×10^{10} p.f.u. ml⁻¹)²². The resulting 2.2-kb fragment was cleaved with *EcoRI* and *KpnI* at an internal site and cloned into the Bluescript plasmid vector (Stratagene). Purified *EcoRI*–*KpnI* fragment was subsequently labelled with random hexamers and used to screen 2×10^5 recombinants from a rat olfactory cDNA library. Filters were washed at low stringency ($2 \times$ SSC buffer, 1% SDS at 55 °C) as previously described². About 100 hybridizing plaques were obtained and several were analysed further. DNA sequence was determined from both strands using double-stranded templates and specific oligonucleotide primers.

§ To whom correspondence should be addressed.

b

Olfactory Channel						Photoreceptor Channel					
Human		Rat		Mouse		Human		Rat		Mouse	
RI	HIII	RI	HIII	BamHI	RI	HIII	RI	HIII	BamHI	RI	HIII
											

METHODS. Each lane of the RNA blot analysis contains 10 μ g total RNA. Membranes were prepared and hybridized as previously described²³ with ³²P-labelled *Eco*RI fragment derived from the full-length cDNA clone. Blots were probed for ribosomal RNA to ascertain that equal amounts of RNA were placed in each lane. For genomic DNA analysis, each lane contained 10 μ g digested DNA that was transferred to Hybond membrane (Amersham) and hybridized under the same conditions used to identify the initial olfactory clone (Fig. 1).

a

1 nA

1 mM

cA cG cA+cG cC

20 s

b

Normalized current

1

10^{-1}

10^{-2}

10^{-1} 1 10 10^2 10^3

Ligand concentration (μM)

cG

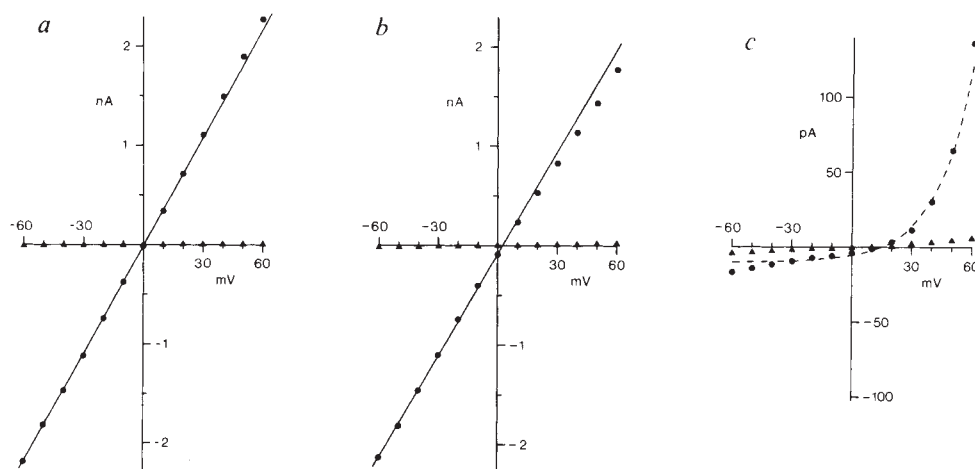
cA

cC

Detailed description: Figure 1 consists of two panels. Panel a is a time-course plot of current (nA) versus time (s). It shows four pulses of current corresponding to the application of cAMP (cA), cGMP (cG), a mixture of cAMP and cGMP (cA+cG), and cAMP alone (cC). The current pulses are approximately 1 nA in amplitude and 20 s in duration. Panel b is a log-log plot of normalized current versus ligand concentration (μM). The y-axis ranges from 10^{-2} to 1, and the x-axis ranges from 10^{-1} to 10^3 . Three data series are shown: cGMP (open circles), cAMP (open triangles), and cAMP (open squares). The cGMP series shows a higher affinity for the channel than the cAMP series. The cAMP series shows a lower affinity for the channel than the cGMP series.

of the order of 10 GΩ. Solutions were at pH 7.6, and contained 10 mM glucose plus the following compositions. Ringer's solution (solution 1): 140 mM NaCl, 5 mM KCl, 10 mM sodium HEPES, 2 mM CaCl₂, 1 mM MgCl₂; Ringer's solution without divalent cations (solution 2): 140 mM NaCl, 5 mM KCl, 10 mM sodium HEPES, 0.5 mM sodium EGTA, 0.5 mM sodium EDTA; pseudointracellular solution (solution 3): 145 mM KCl, 10 mM sodium HEPES, 0.5 mM sodium EGTA, 0.5 mM sodium EDTA. Cyclic nucleotides were bath-applied using a solenoid-controlled rotary valve system²⁴.

FIG. 4 Steady-state current-voltage relations for the expressed channel under different ionic conditions. In all three cases 1,000 μM cA was present, which fully saturated the current. The experiment consisted of making 1-s voltage pulses at ± 10 mV increments from a holding potential of zero, and the current at each voltage was measured at the end of the voltage pulse. $\blacktriangle$, Relation obtained in the absence of ligand (that is, background current); $\bullet$, relation induced by ligand (that is, with the background relation already subtracted). *a*, Ringer's solution without divalents (see solution 2 in Fig. 3 legend) on both sides of membrane. Straight line is fitted to points at negative voltages and extrapolated to positive voltages. *b*, Ringer's solution without divalents in pipette and pseudointracellular solution (solution 3 in Fig. 3 legend) in bath. Again, the straight line is fitted to points at negative voltages and extrapolated to positive voltages. Both *a* and *b* represent results from the same patch as



patch as in Fig. 3*a*. From two patches, the average half-saturating concentration ($K_{1/2}$) values at +40 mV were 1.2 μM (cG), 38 μM (cA) and 88 μM (cC), and the Hill coefficient was 1.9; at -40 mV, the $K_{1/2}$ values were about 10% higher, but the Hill coefficient was similar. From 12 other patches with physiological concentrations of divalent cations (solution 1 in Fig. 3 legend) on the extracellular side of the membrane, the $K_{1/2}$ values at +40 mV were 2.4 ± 1.1 μM (cG), 68 ± 38 μM (cA) and 123 ± 32 μM (cC), respectively, and the Hill coefficient was 2.0 ± 0.2 ; at -40 mV, the $K_{1/2}$ values were about 40% higher. These numbers are again broadly consistent with findings from the native channel in olfactory cilia⁴, except that the $K_{1/2}$ ratio between cA and cG is about two for the native channel, compared with ~ 30 for the expressed channel. At nonsaturating ligand concentrations, no desensitization or inactivation of the expressed channel was observed up to 20 s after ligand application at either positive or negative voltages. Finally, *l*-cis-diltiazem¹⁴ at 0.5–1 mM reduced by half the current activated by 100 μM cAMP (four experiments, data not shown).

The current-voltage relation (filled circles) obtained at saturating (1 mM) cA concentration and with symmetrical Ringer's solutions without divalent cations is shown in Fig. 4*a*. Identical relations were obtained with 1 mM cG or cC. The relation is almost linear; the very slight upward curvature probably reflects a small increase in the open probability of the liganded channel at positive voltages, as has been described for the rod channel^{15,16}. Figure 4*b* shows, in the same patch, the current-voltage relation when most Na^+ in the bath solution (that is, cytoplasmic side of the membrane) was replaced by K^+ (solution 3, Fig. 3 legend). The current at negative voltages is practically identical to that in Fig. 4*a*, but the outward current at positive voltages is smaller, attributed to a slightly smaller conductance to K^+ . The reversal potential in Fig. 4*b* is +3 mV, with a mean of +4.5 mV from two experiments. On the basis of the Goldman-Hodgkin-Katz equation¹⁷, this reversal potential gives a $P_{\text{K}}/P_{\text{Na}}$ permeability ratio of 0.82, similar to that observed for the native channel^{4,18}. Figure 4*c* shows the current-voltage relation, from a different patch, with physiological Ringer's solution with divalent cations (solution 1) in the patch pipette and pseudointracellular solution (solution 3) in the bath. The relation is nearly linear at negative voltages, but shows outward rectification at voltages positive to the reversal potential, reflecting a voltage-dependent block by divalent cations. The overall relation can be roughly described by a scaling of the function $j(V) = e^{(V-V_r)/V_0}$ (dashed curve), where V_r is the reversal potential and V_0 is the slope constant. From four experiments, V_r was 13 ± 2 mV and the best-fit V_0 values was 18 ± 1 mV.

in Fig. 3. *c*, Ringer's solution with divalents (solution 1 in Fig. 3 legend) in pipette and pseudointracellular solution in bath. Different patch from *a* and *b*. Dashed curve is a scaling of the equation $j(V) = e^{(V-V_r)/V_0}$ (see text).

This relation with a similar V_0 also describes the behaviour of the cyclic-nucleotide-gated channel in rods under similar ionic conditions⁶. The above characteristics are consistent with observations from the odorant-regulated channel in intact olfactory neurons^{18,19}, although excised patches from native ciliary membranes⁴ give a fairly linear current-voltage relation even at positive voltages.

In summary, the primary structure of a cyclic-nucleotide-gated channel from olfactory neurons has been deduced by cloning and DNA sequence analysis. The homology between this channel and the cyclic-nucleotide-gated channel in retinal rods is consistent with their similar physiological properties. The 30–50-fold higher sensitivity of the olfactory channel⁴ to cyclic nucleotides compared to the rod channel^{12,13} may result from the amino-acid differences in the putative nucleotide-binding site of the two proteins, though this remains to be examined. We consistently found a $K_{1/2}$ for cAMP of ~ 40 μM , but the $K_{1/2}$ for cAMP reported by Nakamura and Gold⁴ was, curiously, bimodal, being ~ 2 μM and 40 μM , respectively. Modification of the expressed or the native channel could result in altered nucleotide affinities. In principle one might expect a high $K_{1/2}$ value to be physiologically appropriate, in that this allows the olfactory sensory neuron to be at rest at basal levels of cAMP, and excited when the cAMP concentration rises substantially in response to odorants. $\square$

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Separation of pluripotent haematopoietic stem cells from spleen colony-forming cells

Richard J. Jones, John E. Wagner, Paul Celano, Milada S. Zicha & Saul J. Sharkis

Johns Hopkins Oncology Center, The Johns Hopkins Medical Institutions, Baltimore, Maryland 21205, USA

LONG-TERM reconstitution of the lymphohaematopoietic cells of a mouse after lethal irradiation requires the transplantation of at least $(5-10) \times 10^3$ bone marrow cells^{1,2}. Several cell-separation techniques based on cell-surface characteristics have been used in attempts to identify the pluripotent haematopoietic stem cells (PHSC), and have allowed the long-term engraftment of lethally irradiated mice with an enriched fraction of fewer than 200 marrow cells³⁻⁵. But these techniques enrich not only for PHSC but also for haematopoietic progenitors, especially day-12 spleen colony-forming units (CFU-S)³⁻⁵. Although day-12 CFU-S have been postulated to be primitive multipotential haematopoietic progenitors, with day-8 CFU-S representing later, more committed progenitors⁶, recent evidence suggests that neither of these CFU-S represents mouse PHSC⁷⁻⁹. Here we report that counterflow centrifugal elutriation, which sorts cells on the basis of size and density, can separate PHSC from these less primitive progenitors. The fraction containing the largest cells was enriched for the granulocyte-macrophage colony-forming units (CFU-GM), but gave only transient, early engraftment and was therefore depleted of PHSC. The intermediate fraction was enriched for CFU-S, but depleted of CFU-GM. Despite being devoid of CFU-GM and CFU-S, the fraction consisting of only morphological lymphocytes gave sustained, albeit delayed, reconstitution of all lymphohaematopoietic cells, and was therefore enriched for PHSC. We conclude that there are two vital classes of engrafting cells: committed progenitors, which provide initial, unsustained engraftment, and PHSC, which produce delayed, but durable, engraftment. Therefore for late haematological reconstitution, PHSC must be transplanted with a distinguishable source of early engrafting cells, thereby allowing the lethally irradiated host to survive initial aplasia.

Counterflow centrifugal elutriation (CCE), which sorts cells on the basis of size and density, can enrich for haematopoietic progenitors in mice and humans^{10,11}. In addition, CCE is an effective technique for depleting T lymphocytes from allogeneic bone marrow grafts as prophylaxis against graft-versus-host disease¹¹. Like other methods of T-lymphocyte depletion, CCE is associated with an increased risk of graft failure, despite

producing a marrow graft enriched for assayable haematopoietic progenitors¹¹. Graft failure after T lymphocyte-depleted allogeneic bone marrow transplantation (BMT) has been considered to result from immunological graft rejection^{12,13}. As mouse PHSC may resemble lymphocytes^{14,15} and seem to display T-lymphocyte antigens on their surfaces^{3,5}, graft failure following some methods of depleting T lymphocytes from marrow grafts may partly be the consequence of also eliminating PHSC. Similarly, early human haematopoietic progenitors, capable of generating long-term marrow cultures while relatively depleted of CFU-GM, have been found in the lymphoid fraction when marrow is separated by light-scattering properties^{16,17}. Therefore, the PHSC could migrate with lymphocytes during CCE and be separable from other haematopoietic progenitors.

We studied the progenitor content and engraftment potential of mouse bone marrow cell fractions sorted by CCE. With the centrifuge rotor speed held at 1,260g, cells were sorted by changing the rate of medium flow. The progenitor content of the various CCE fractions is shown in Table 1. The cells remaining in the chamber after CCE (the 'rotor-off' fraction) were enriched fourfold for CFU-GM and about twofold for both day-8 and day-12 CFU-S. This fraction contained a high percentage of large blast cells and was virtually depleted of lymphocytes. The cells collected at a flow rate of 29 ml min⁻¹ (FR 29) were intermediate in size. This fraction was enriched threefold for CFU-S (both day-8 and day-12), but relatively depleted of CFU-GM. Virtually all the cells collected at a flow rate of 25 ml min⁻¹ (FR 25) were morphologic lymphocytes. This fraction was almost depleted of CFU-GM and CFU-S (both day-8 and day-12). The absence of assayable CFU-GM and CFU-S in the FR 25 cells is unlikely to be the result of altered accessory cell numbers in that fraction, as all the CFU-GM and CFU-S present in the unfractionated marrow can be accounted for in the other CCE fractions.

The engraftment potential of the CCE fractions was studied by transplanting lethally irradiated female B6D2F₁ mice with male B6D2F₁ marrow cells (Table 2). Cells from bone marrow, spleen and thymus were collected at various time intervals after BMT, and the origin of the engrafted cells was determined by DNA hybridization with a Y chromosome-specific probe^{7,18}. Female mice transplanted with 2×10^4 rotor-off cells showed definite early engraftment with 570 ± 70 circulating neutrophils per ml, and bone marrow cells that were completely donor, 14 days after BMT. However, nearly half of the mice transplanted with the rotor-off cells died later after BMT than the radiation controls, and the bone marrow of the surviving mice was only $11 \pm 2.3\%$ donor phenotype two months after BMT. The rotor-off cells did not produce durable engraftment, despite the fact that 2×10^4 rotor-off cells contain about the same number of CFU-GM as 10^5 unfractionated marrow cells (Table 1), which produce complete, durable engraftment in 100% of lethally irradiated mice^{1,19}. Therefore, the rotor-off cells were relatively depleted

TABLE 1 CCE characteristics of mouse bone marrow

Flow rate (ml min ⁻¹)	Unseparated	25	29	33	Rotor-off
% Cell recovery	100	25.5 ± 3.6	12.3 ± 1.2	15.7 ± 3.3	24.9 ± 2.3
% Lymphs/blasts	24 ± 2/5 ± 0.4	99.4 ± 0.3/0	62 ± 14/0	22 ± 3/17 ± 0.4	0.8 ± 0.3/10 ± 1.4
CFU-GM per 10 ⁵ cells	538 ± 31	2.8 ± 0.6	288 ± 66	689 ± 57	2,056 ± 456
Day-8 CFU-S per 10 ⁵ cells	26.4 ± 1.9	0.6 ± 0.2	82 ± 2.5	23.3 ± 0.2	46 ± 7
Day-12 CFU-S per 10 ⁵ cells	28.2 ± 2.8	0.4 ± 0.1	75 ± 6.2	23.5 ± 0.4	46.5 ± 7

CCE was performed using a Beckman J-6M centrifuge with a JE-6B elutriator rotor and a standard chamber (Beckman Instruments, Palo Alto, California). One master-flex peristaltic pump (Cole Palmer, Chicago, Illinois), equipped with a potentiometer, provided precisely metered flow of medium. Marrow cells were loaded into the chamber ($\sim 2 \times 10^8$) at a flow rate of 15 ml min⁻¹ and a rotor speed of 1,260g. About 20% of the cells (mostly crenated cells, small lymphocytes and nucleated red cells) were eluted while the cells were being loaded into the chamber; these cells contained no assayable progenitors. With the rotor speed held constant, the cells were eluted by changing flow rates, collecting 300 ml at each flow rate. The cells remaining in the chamber at the end of CCE were collected as the rotor-off fraction. The data are presented as the mean ± s.e.m. of seven separate experiments. CFU-S and CFU-GM were assayed as previously described¹, except that the CFU-GM cultures used enriched conditions containing 30% fetal calf serum, 1% BSA and 10^{-4} M 2-mercaptoethanol. The CFU-S results are the actual number of spleen colonies counted, without correction for the fraction seeding the spleen (*f*-factor).

TABLE 2 Engraftment after BMT with CCE fractions

CCE fraction	No. of cells transplanted	Mice surviving (60 days)	Phenotype of engraftment	% Male cells day-14	% Male cells day-60
Rotor-off	2×10^4	15 of 28	Bone marrow	98 ± 1.9	11 ± 2.3
FR 25	1×10^5	0 of 16	—	—	—
FR 25	2×10^4 (male)	22 of 30	Bone marrow	0	51 ± 15
+	+		CFU-GM	—	81 ± 12
"Rotor-off"	2×10^4 (female)		B cells	—	54 ± 12
			T cells	—	77 ± 10

Data are presented as the mean $\pm$ s.e.m. of at least three separate experiments with two mice per experiment killed at day 14 and day 60 after BMT (total of 6–8 mice examined for each data point) for Y chromosome determinations by DNA hybridization of the reconstituted haematological lineages. CFU-GM were cultured from the bone marrow, plucked and pooled (about 500–2,000 colonies per mouse) for Y chromosome determination. B cells were recovered from the spleen by indirect immune adherence (panning) using the technique of Engleman²⁰. Plastic-nonadherent spleen cells treated with anti-B220 monoclonal antibody²¹ were placed in anti-rat IgM-coated Petri dishes (10^7 cells ml⁻¹) for 45 min at 4 °C. Unbound cells were removed by rocking and gentle aspiration; the adherent B220-positive B cells were collected by vigorous pipetting. Thymus glands were the source of T cells. Standard procedures were used in the preparation of DNA samples²². DNA (2 μ g) was boiled in 0.4 N NaCl and immobilized on nylon membranes. The DNA was hybridized with an α -³²P-labelled mouse Y chromosome-specific fragment (pY2)^{7,18}. To quantitate the relative contributions of male and female cells in each sample, the experimental DNA was slotted in parallel with DNA from known mixtures of male and female marrow cells as described by Lemischka *et al.*²³. The DNA was also probed with a β -actin probe to correct for DNA loading differences, and blots were scanned densitometrically. The mean day of death after BMT: 16 ± 0.9 , mice transplanted only with rotor-off cells; 11.8 ± 0.5 , mice transplanted with FR 25 cells; 11.6 ± 1.0 , mice transplanted with FR 25 and rotor-off cells; and 12 ± 0.2 , radiation-only control mice.

of PHSC, even though this CCE fraction was enriched for committed progenitors and produced early engraftment.

The transplantation of 10^5 FR 25 cells would not rescue the mice from lethal aplasia (Table 2). We have previously suggested that there are two essential phases of engraftment following BMT: an early phase resulting from committed progenitors, and a delayed phase owing to PHSC (ref. 7). We therefore transplanted 2×10^4 female rotor-off cells, to provide an early wave of transient engraftment, mixed with 2×10^4 male FR 25 cells. This number of FR 25 cells contained virtually no detectable CFU-GM or CFU-S. Although the reconstituted recipient mouse bone marrow was completely derived from the female rotor-off cells 14 days after BMT, all haematological lineages were primarily derived from the male FR 25 cells two months after BMT (Table 2). Thus, the FR 25 cells, which were nearly completely depleted of committed progenitors (including day-12 CFU-S) and could not rescue mice from lethal aplasia, contained the PHSC.

Our data demonstrate that neither day-8 CFU-S, nor the possibly more primitive day-12 CFU-S, represent the PHSC. Thus, durable reconstitution of all lymphohaematopoietic lineages following marrow ablative therapy remains the only way to identify the PHSC. However, the PHSC proliferate slowly and produce only delayed engraftment. Therefore, to demonstrate late engraftment from transplanted PHSC, these cells must be combined with a distinguishable source of early engrafting cells (committed progenitors), which will allow the marrow-ablated host to survive initial aplasia. □

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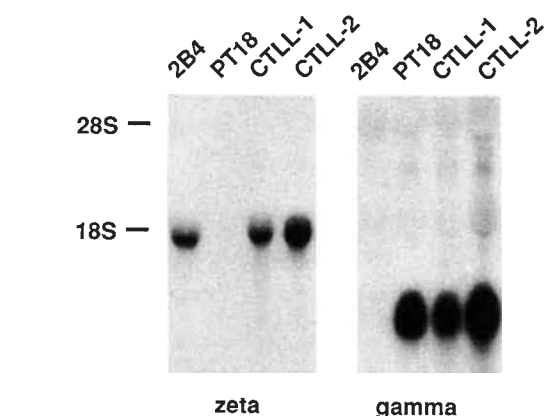
Family of disulphide-linked dimers containing the ζ and η chains of the T-cell receptor and the γ chain of Fc receptors

David G. Orloff, Chisei Ra*, Stuart J. Frank, Richard D. Klausner & Jean-Pierre Kinet*

Cell Biology and Metabolism Branch, National Institute of Child Health and Human Development and Molecular Allergy and Immunology Section*, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20892, USA

STIMULATION of T cells by antigen activates many signalling pathways¹. The capacity for this range of biochemical responses may reside in the complex structure of the seven-chain T-cell antigen receptor (TCR)². In addition to the complexity shared by all TCRs, coexpression of zeta (ζ) and the distinct but related eta (η) chain creates structural diversity among the TCR complexes expressed on a given cell^{3,4}. In most murine T cells that we have studied, about 90% of the heptameric receptor complexes contain a $\zeta\zeta$ disulphide homodimer, whereas 10% contain a $\zeta\eta$ disulphide heterodimer⁵. Recent studies suggest that ζ has a critical role in allowing antigen to activate the cell, whereas η expression has been correlated with the capacity for antigen-induced phosphoinositide turnover^{6,7}. A third ζ -related protein, the gamma (γ) chain of the Fc ϵ and some Fc γ receptors, exists as a disulphide homodimer in those complexes^{8–10}. The structural relatedness of ζ and γ is emphasized by the recent demonstration of $\zeta\zeta$ in association with CD16 in TCR-negative natural killer cells^{11–13}. Here we identify T cells lacking Fc receptors but coexpressing ζ , γ , and η , document the formation of novel heterodimers between ζ and γ and between η and γ and show their association with the TCR. A greater range of homologous coupling structures than previously thought may be one way of achieving the variety of TCR-mediated (and possibly Fc receptor-mediated) biochemical responses and effector functions.

Total cytoplasmic messenger RNA was prepared from the mast cell line PT18, the T-cell hybridoma 2B4, and from the cytotoxic T-cell line CTLL, including clones expressing low (CTLL-1) and high (CTLL-2) levels of surface CD3 antigen, as assessed by fluorescence activated cell sorting (FACS) (not shown). Northern blot analysis (Fig. 1) using complementary



◀ FIG. 1 Northern-blot analysis of TCR ζ and Fc ϵ R γ -chain mRNA levels in mast cells and T cells. Total cytoplasmic RNA from the indicated cell lines was prepared by a modification of the method of Favolaro¹⁸. RNA (15 μ g) was electrophoresed through 1.2% agarose (containing formaldehyde) and then transferred to Nytran (Schleicher & Schuell) membranes. Replicate blots were probed with an *Sst*I–*Eco*RI fragment (~320 base pairs (bp)) of the murine ζ cDNA¹⁴ (zeta) or with a *Hind*III–*Eco*RI fragment (~420 bp) of the murine γ cDNA¹⁵ (gamma), each labelled by random priming with [³²P]dCTP (Oligolabelling Kit, Pharmacia). Blots were washed at 55 °C and 0.2 $\times$ SSC before autoradiography.

DNA probes for murine ζ (ref. 14) and γ (ref. 15) reveals γ message alone in PT18, ζ alone in 2B4, and both in CTLL.

We next investigated the expression and dimerization of ζ -family proteins. CTLL cells were metabolically labelled with [³⁵S]cysteine and [³⁵S]methionine ([³⁵S]Cys/Met), solubilized and immunoprecipitated using an anti- ζ -peptide antibody, which is known to cross-react with η (anti- ζ/η)³.

Figure 2a shows the results of two-dimensional nonreduced-reduced SDS-PAGE (2D-diagonal PAGE) separation and autoradiography. Four disulphide dimers, involving species with apparent relative molecular mass 7,000, 16,000 and 22,000 (M_r , 7K, 16K and 22K), and migrating at 23, 29, 32 and 38K in the nonreduced dimension, were specifically immunoprecipitated with anti- ζ/η antibody. We definitively identified the 16 and 22K species as ζ and η , respectively, by immunoblotting with anti- ζ/η after 2D-diagonal PAGE separation of unlabelled precipitates (Fig. 3). In 2B4 cells (Fig. 3a), $\zeta\zeta$ (falling from 32K in the nonreduced dimension) and $\zeta\eta$ (falling from 38K) are

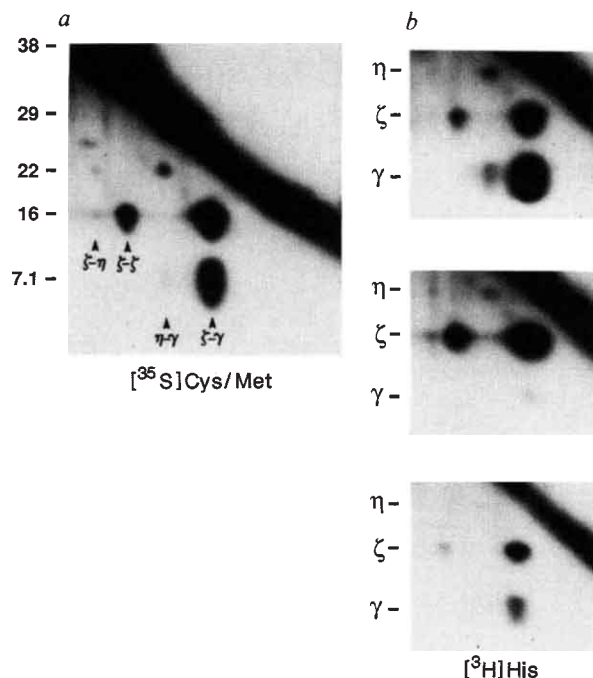


FIG. 2 Expression and dimerization of ζ -family proteins in CTLL cells and analysis of amino-acid composition by metabolic labelling. *a*, Cells labelled with [³⁵S]cysteine and [³⁵S]methionine (M_r markers (K) on the left); *b*, cells labelled with single amino acids as indicated. RPMI 1640 medium was prepared (Selectamine Kit, GIBCO) free of the indicated amino acid(s) and supplemented with 5% FCS. Cells (5×10^7) were collected, washed in PBS, and depleted of the amino acid(s) chosen for labelling by incubation at 37 °C for 15 min in the appropriate medium. Cells were pelleted and resuspended in the same medium (10^7 ml^{-1}) with added labelled amino acid(s) (0.5 mCi ml^{-1} : [³⁵S]cysteine, [³H]histidine (Amersham); [³⁵S]methionine (ICN)) and were incubated for 2 h at 37 °C. After labelling, cells were pelleted, lysed as described previously² in buffer containing Tris buffer (50 mM pH 7.4), 300 mM NaCl, and 0.5% Triton X-100, protease inhibitors, and 10 mM iodoacetamide (Triton lysis buffer). The postnuclear supernatants were immunoprecipitated using anti- ζ -peptide 3 serum, which crossreacts with η (anti- ζ/η)³ bound to protein A agarose (BRL). The precipitated proteins were separated by 2D-diagonal PAGE (first dimension, 13% acrylamide tube; second dimension, 14% Acrylaide slab) as described previously². Gels were fixed, treated with sodium salicylate (1M), dried, and autoradiographed at -80 °C. Signal intensity was measured by one-dimensional densitometry using an LKB laser-enhanced densitometer. From cDNA sequence, ζ is predicted to have three methionines, one cysteine, two histidines; γ has no predicted methionines, two cysteines, and one histidine; η has two methionines, two cysteines and two histidines^{4,14,15}.

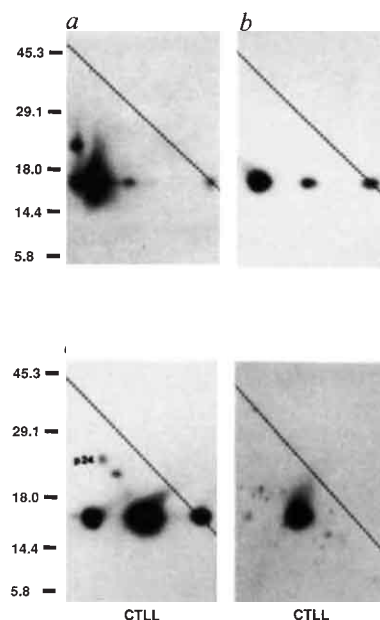


FIG. 3 Identification of ζ family dimers by western blotting and demonstration of their association with the TCR. 2B4 and CTLL cells (10^8 per sample) were solubilized in Triton lysis buffer and NK3.3 cells (5×10^7 per sample) were lysed in buffer containing 1% digitonin, 150 mM NaCl, 50 mM HEPES, protease inhibitors, and 10 mM iodoacetamide (digitonin lysis buffer). The postnuclear supernatants were immunoprecipitated with anti- ζ/η serum (*a*, *b*, *c*) or with anti-CD3 ϵ monoclonal antibody (*d*), and the proteins separated by 2D-diagonal PAGE (first dimension, 13% acrylamide tube; second dimension, 14% acrylamide slab). Proteins were transferred electrophoretically to nitrocellulose. Immunoblotting was carried out as described previously⁵ using anti- ζ/η serum at a 1:200 dilution in 2.5% milk in PBS. The monomeric ζ on the diagonals of the anti- ζ/η precipitates is thought to be an artefact of solubilization. The identity of the immunoreactive 24K species (p24) is unknown. The ζ species falling from 27K in the 2B4 panel (*a*) is consistently seen and is thought on the basis of pulse-chase and steady-state metabolic labelling experiments to have been linked in the first dimension to a partially degraded ζ that has lost its immunoreactivity (D.G.O and R.D.K., unpublished results). M_r markers (K) on the left.

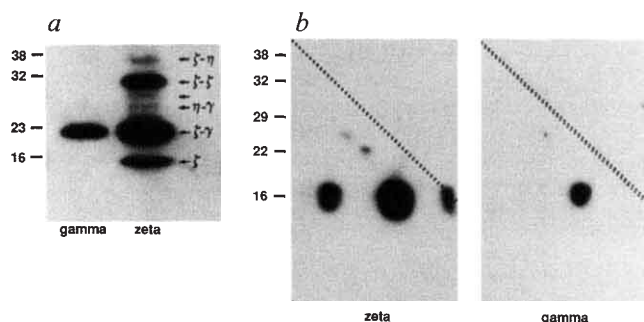


FIG. 4 The 7K partner of ζ and η in CTLL is true FcR γ -chain. Lysates of CTLL cells (5×10^7 cells per condition) were immunoprecipitated with anti- ζ/η (zeta) or with anti- γ (gamma) antibodies. The latter were raised in rabbits using a unique peptide from the cytoplasmic tail of human γ coupled through a terminal cysteine to chicken serum albumin using Sulfo-MBS (Pierce). Immunoprecipitated proteins were separated by nonreducing SDS-PAGE (a) or by 2D-diagonal SDS-PAGE (b), transferred to nitrocellulose, and immunoblotted with anti- ζ/η serum. The ζ monomer and ζ -family dimers are labelled in a. The unlabelled arrow indicates the 31K dimer that includes p24. The incomplete precipitation of $\zeta\gamma$ and $\eta\gamma$ is due to the low titre of the antibodies used.

readily detected. In CTLL (Fig. 3c), $\zeta\zeta$ and $\zeta\eta$ are also detected, but in addition, prominent ζ and η spots are seen falling from 23 and 29K, respectively. As seen in Fig. 2a (see also Fig. 2b, [^{35}S]Cys), these were both disulphide-linked in the first dimension to a species, not recognized by the anti- ζ/η antibody, of approximately 7K, which is the predicted molecular weight of the γ chain of the FcR complex⁸. Indeed, metabolic labelling with cysteine, methionine, and histidine alone (Fig. 2b) reveals that for each amino acid the ratio of incorporation into ζ to that into the 7K species is consistent with the latter's being γ ^{14,15}.

The 7K partner of ζ and η was definitively identified as the FcR γ -chain by antibodies raised against a unique cytoplasmic peptide of human γ . Anti- ζ/η and anti- γ precipitates of equal amounts of CTLL lysate were separated by nonreducing SDS-PAGE (Fig. 4a) or by 2D-diagonal SDS-PAGE (Fig. 4b), the proteins transferred to nitrocellulose, and immunoblotted with anti- ζ/η antibody. The anti- γ immunoprecipitates share only one species with the anti- ζ precipitates. This is the prominent ζ -7K (23K) dimer seen in previous experiments. Thus, the 7K protein is indeed γ . On longer exposures, the $\eta\gamma$ dimer is also seen in the anti- γ precipitates. In metabolic labelling and anti- γ immunoblotting experiments (not shown), a much more abun-

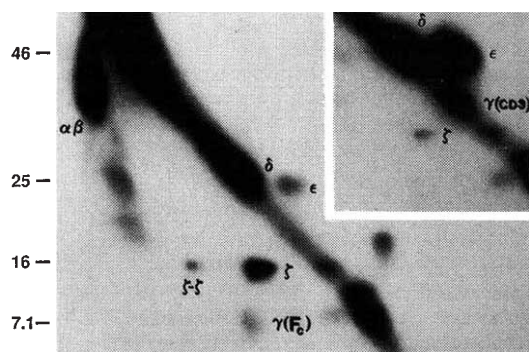


FIG. 5 Most surface TCR complexes on CTLL cells contain $\zeta\gamma$. CTLL cells were washed in ice-cold PBS, and surface-iodinated using lactoperoxidase as described previously². Cells (5×10^7) were solubilized in Triton lysis buffer and immunoprecipitated with anti- ζ/η serum or anti-CD3 ϵ monoclonal antibody (inset). Proteins were separated by 2D-diagonal PAGE; the gels were fixed, dried, and placed on X-ray film at -80°C for 9 days. The off-diagonal 10K species, the 7-12K on-diagonal spots, and the 18-30K streak below TCR $\alpha\beta$ are all nonspecific. M_r markers (K) on the left.

dant species of apparent M_r 14K (nonreduced) is detected in the anti- γ precipitate. This is the $\gamma\gamma$ homodimer.

The formation of $\zeta\gamma$ heterodimers in these cells allows us to propose that the analogous ζ -containing heterodimer noted by Anderson *et al.*^{11,13} in human natural killer (NK) cells is most likely to be ζ - γ . We can infer the existence of the same heterodimer in an anti- ζ/η precipitate from an NK cell line¹⁶ (Fig. 3b), which expresses γ message¹⁷. After digitonin lysis of these cells, both $\zeta\gamma$ and $\zeta\zeta$ can be coprecipitated with CD16 (not shown).

We next investigated whether any of these novel heterodimers could be found on the surface of CTLL, which is CD3 $^+$, FcR $^-$ by FACS. Figure 5 shows the results of surface iodination of CTLL-2, anti- ζ/η immunoprecipitation followed by 2D-diagonal PAGE analysis, and reveals that $\zeta\gamma$ and $\zeta\zeta$ are expressed on the cell surface. The 40-45K TCR $\alpha\beta$ heterodimer as well as the CD3 ϵ - and δ -chains are clearly coprecipitated with the anti- ζ/η antibody, though the inferred associations might well be through $\zeta\zeta$ and not $\zeta\gamma$. Indeed, anti-CD3 ϵ immune precipitates of surface-labelled CTLL cells (Fig. 5, inset) contain predominantly one ζ species. From its position relative to CD3 ϵ , however, and, more important, the point from which it falls off the diagonal, the ζ species seen is clearly not $\zeta\zeta$ but rather part of the $\zeta\gamma$ heterodimer, though γ cannot be seen at this exposure. Thus, most of the TCRs on the surface of CTLL cells are of a novel structural class containing the $\zeta\gamma$ dimer. No $\gamma\gamma$ was detectable within the TCR complex on these cells. When anti-CD3 ϵ immunoprecipitates of unlabelled CTLL lysates were analysed by 2D-diagonal anti- ζ/η immunoblotting (Fig. 3d), we could show the association with the TCR of $\zeta\gamma$, $\zeta\zeta$, and, on longer exposures (not shown), $\eta\gamma$. Finally, comparison, using equal amounts of CTLL lysate, of precipitation with anti- ζ/η (Fig. 3c) to that with anti-CD3 ϵ (Fig. 3d) reveals that $\zeta\gamma$ is found in association with CD3 in these cells to the exclusion of $\zeta\zeta$, the principal TCR-associated disulphide dimer in all other T cells studied so far.

We have identified two novel heterodimers involving members of the ζ -family of proteins. Furthermore, we have shown surface expression of $\zeta\gamma$ and its exclusive association with the TCR complexes on the cells studied. Although the capacity of this new type of TCR to transduce signals has yet to be studied in detail, in CTLL cells, anti-receptor antibody stimulation initiates phosphoinositide hydrolysis and cellular proliferation, presumably through production of interleukin-2 (B. B. Niklinska & J. D. Ashwell, personal communication). Future studies will show whether the now expanded roster of ζ family dimers distinguishes signalling classes of TCR as well as FcR. Whether this structural diversity will shed light on the control of specific effector functions mediated by these receptors will also be of great interest. □

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Microconversion between murine *H-2* genes integrated into yeast

Christopher J. Wheeler^{*†}, Daniel Maloney[‡],
Seymour Fogel^{‡§} & Robert S. Goodenow^{*§}

^{*} Department of Molecular and Cell Biology and [‡] Department of Plant Biology, University of California, Berkeley, California 94720, USA

PATCHWORK homology observed between divergent members of polymorphic multigene families is thought to reflect evolution by short-tract gene conversion (nonreciprocal recombination), although this mechanism cannot usually be confirmed in higher organisms¹⁻¹². In contrast to meiotic conversions observed in laboratory yeast strains, apparent conversions between polymorphic sequences, such as the class I loci of the major histocompatibility complex (MHC), are short and do not seem to be associated with reciprocal recombination (crossover, exchanges)^{7-9,13-23}. We have now integrated two nonallelic murine class I genes into yeast to characterize their meiotic recombination. We found no crossovers between the MHC genes, but short-tract 'microconversions' of 1-215 base-pairs were observed in about 6% of all meioses. Strikingly, one of these events was accompanied by a single base-pair mutation. These results underscore both the importance of meiotic gene conversion and sequence heterology in determining conversion patterns between divergent genes.

Haploid yeast strains each carrying one of two class I gene integrants were crossed and the resulting diploids sporulated. A total of 378 unselected tetrads were examined genetically. Tetrads with a single reciprocal exchange between the class I genes will contain three arginine prototrophic spores (Fig. 1). The 58 tetrads in the sample containing three arginine prototrophs were therefore subjected to Southern blot analysis monitoring five segregating restriction site differences between the parental genes (Fig. 1). No exchanges within the class I genes were found. Thus, we conclude that there were no detectable exchanges between these genes in the entire sample. However, we did detect three long-tract conversions among these tetrads, extending at least 2.5, 1.35 and 2.28 kilobases (kb) (Fig. 2). In two of these, the *Arg4* gene was coconverted and the third had an independent conversion of *Arg4*. Southern blot analysis of 150 of the 320 tetrads in which *Arg4* segregated 2:2 revealed no additional long-tract conversions (<0.66%).

As Southern blot analysis will not reveal short-tract conversions that do not alter a restriction site, we also performed RNase protection analysis using locus-specific probes to identify microconversions on class I DNA rescued from individual ascospore colonies. In these experiments, the 58 tetrads with three arginine prototrophic spores revealed no microconversions; but in 68 tetrads not previously screened by Southern blotting, we found four tetrads containing alterations in class I DNA. The altered class I genes were sequenced to identify the nature of the mismatch, and in all four tetrads we found short conversion tracts ranging from 1-215 nucleotides, which corresponded to sequences from the other parental class I gene (Fig. 3). Three tetrads segregated 3:1 for the class I sequence, indicating that the event occurred after meiotic DNA replication, whereas the remaining tetrad segregated 4:0, indicating a pre-meiotic origin. As the four microconversion events occurred in a sample of 68 tetrads in which *Arg4* segregated 2⁺:2⁻, we estimate that about 6% of the total sample of tetrads contained short-tract conversions. This is a minimum estimate, as our probes covered only 50% of the class I genes. We emphasize that as no microconversions were identified in the tetrads con-

taining three arginine prototrophs, these events show no association with reciprocal exchange.

From the analysis of yeast crosses involving a single heterozygous site²³, it is generally accepted that conversion is not associated with mutation. It was therefore surprising that one of the convertants (CON5) was associated with a single base

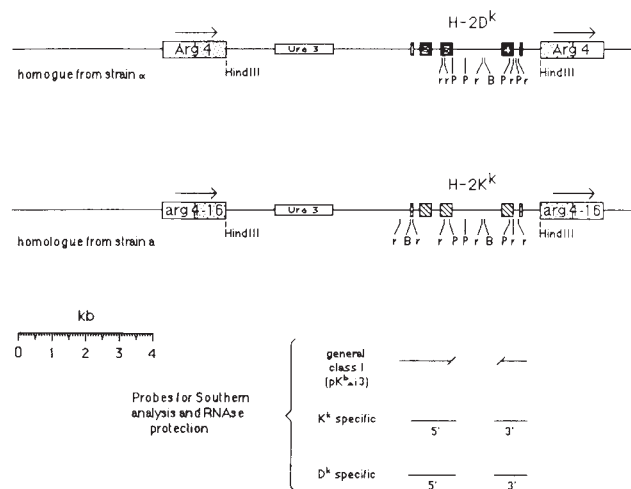


FIG. 1 Configuration of class I genes in the diploid MHC-transformed yeast strain. Transcriptional orientation of the class I and *Arg4* genes are depicted by arrows, and class I exons are represented by solid (*H-2K*) or hatched (*H-2D*) boxes. Polymorphic restriction sites shown are *Bam*HI (B), *Pst*I (P) and *Rsa*I (r). Pre-meiotic DNA replication in this diploid should yield four chromosomes, two (containing *H-2D*) capable of conferring arginine prototrophy and two (containing *H-2K*) conferring arginine auxotrophy. A crossover between the *H-2* loci would yield two recombinant chromatids each carrying one copy of *ARG4*⁺ and one of *arg4-16*. Thus, any tetrad in which such a crossover has occurred will contain three arginine prototrophic spores and one auxotroph. Other events, such as crossovers in the homozygous *Yip5* sequences or conversion of *arg4-16* to *ARG4*⁺, will also generate tetrads with three arginine prototrophs. Thus, to identify crossovers within class I DNA, all four spores in each tetrad containing three prototrophs were analysed as described below.

METHODS. The *H-2K*⁺ and *H-2D*⁺ genes²⁹ were subcloned into *Yip5* (ref. 30) derivatives carrying alleles of the yeast *Arg4* gene. As a result, *H-2K*⁺ was linked to *arg4-16* in plasmid *paK*⁺SG and *H-2D*⁺ was linked to *ARG4*⁺ in *paD*⁺SG. The plasmids included about 400 bp of 5' flanking class I sequence and all class I exons and introns up to the *Bgl*II site in intron 5. The *H-2K*⁺ fragment contained an engineered *Apal* site deletion within exon 4 at position 2,785 (ref. 28), and the *H-2D*⁺ fragment contained a filled-in *Nar*I site within exon 3 at position 799. Targeting to the chromosomal *Arg4* locus on chromosome VIII was achieved by linearization within the plasmid *Arg4* sequences and transformation of the *Arg4*⁺ strain with *paD*⁺SG, and of the *arg4-16* strain with *paK*⁺SG. Orientation and copy number were determined by Southern blot hybridization using the general class I probe *pK*⁺ (ref. 19). Appropriate transformants harbouring the class I genes integrated at *Arg4* were mated to produce a diploid strain heterozygous for class I genes, *Arg4* alleles, and other yeast markers. Insertion of the *H-2* genes did not alter the frequency of meiotic segregation ratios for the linked and unlinked heterozygous yeast markers *thr1*, *put2*, *cdc12*, *SUF8*, *ade6*, *trp1*, *leu2* and *MAT* (data not shown). DNA was isolated from individual ascospore colonies in a procedure modified from Rothstein³¹, and Southern blots were analysed for polymorphic restriction sites using the *pK*⁺ probe. Plasmids containing class I sequences were rescued from individual spore DNA by first cutting 400 ng with *Hind*III, diluting with 400 μ l ligation buffer and ligating with 1 unit T4 DNA ligase before transformation of competent *E. coli*. Rescued plasmids were collected as described previously³¹ and digested with *Sau*3A before RNase protection analysis as previously described¹⁹ using *H-2K*⁺ and *H-2D*⁺-specific probes corresponding to the 5' and 3' ends of each class I gene. Probes for the 5' end were *Bam*HI-*Pvu*II fragments from each gene, extending from the beginning of exon 1 to the beginning of intron 3. Probes for the 3' end were *Spe*I-*Bgl*II fragments, extending from the end of intron 3 to the middle of intron 5. About 50% of the genes were represented in the locus-specific probes. Rescued plasmids were also subjected to direct double-stranded sequencing as described²⁹.

[†] Present address: Department of Medicine, Division of Immunology, Stanford University, Stanford, California 94305, USA.

[§] To whom correspondence should be addressed.

substitution absent in either parental gene, as determined by intra-ascus sequence comparisons. As conversion-associated mutations are also thought to occur within the chicken immunoglobulin gene family²⁴, our observations imply that such mutations may be produced by functionally conserved recombination machinery acting on dissimilar DNA sequences. Furthermore, if these events are common among multigene families evolving through microconversion, an additional level of diversification would be afforded to affected loci relative to that conferred by microconversion alone. In general, it will be important to determine the extent to which mutation accompanies microconversion in yeast.

The observed conversions were not randomly distributed throughout the class I genes. The conversion tracts CON5 (donated by *H-2D^k*) and CON6 (donated by *H-2K^k*) involve the

same stretch of nucleotides. Similarly, one end of CON1 lies close to or at the site converted in CON4; whereas CON7 occurred about 250 bp away from this region. This clustering may reflect the presence of recombination hotspots within the class I genes, as both the short- and long-tract conversion endpoints often mapped near sequences resembling the *Escherichia coli* recombination sequence, *chi* (Fig. 2).

In terms of the mechanism involved in short-tract conversions, the multiple, clustered mismatches resulting from heterozygosity between divergent genes may prevent the extension of hybrid DNA tracts in an early stage of recombination resulting in microconversions. But because closely spaced heterozygosities are required to detect microconversion, short-tract events within regions of high sequence identity may go undetected. In any case, the lack of exchange associated with microconversion may reflect a requirement for more extensive heteroduplex formation before exchange can occur^{25,26}. By contrast, the less frequent long-tract conversions we observed may be similar to more conventional meiotic conversions in yeast^{20,24}.

The microconversions reported here provide evidence that bona fide gene conversion is responsible for the intrachromosomal germ-line mutations identified in mice and underlies the diversification of multigene families. In fact, CON5 and CON6 overlap with the maximum possible sequence transfer in the mouse *H-2K^{km2}* convertant¹⁹. The apparent discrepancy between microconversion in yeast, which involves *H-2K^k* and

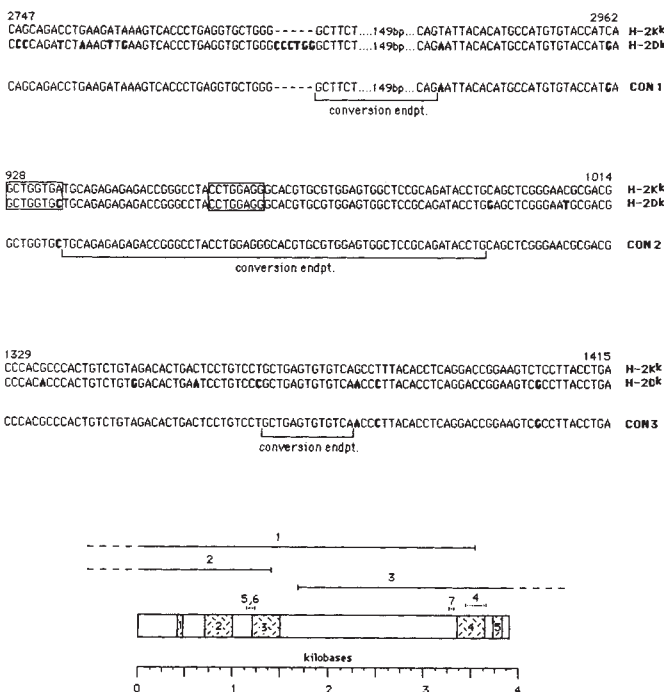


FIG. 2 Position and nucleotide sequence of MHC class I long-tract conversions in yeast. Southern blotting and RNase protection revealed three long-tract conversions (0.67% of unselected tetrads). Their positions are depicted above a diagram of a class I gene (bottom). Long conversion tracts are labelled 1, 2 and 3 in the schematic and the sequences around the class I endpoints (endpt.) are depicted above as CON1, CON2 and CON3, along with the parental sequences. Dashes indicate an uncharacterized terminus. CON1 was donated by *H-2K^k*. The same tetrad also harbours an apparently independent conversion of *Arg4*. One endpoint lies in exon 4, between nucleotide positions 2,794 and 2,945 (ref. 28) and falls within the region of greatest sequence similarity between *H-2D^k* and *H-2K^k*, a span of 149 identical base pairs. This region includes two sequence motifs bearing marked similarity (one copy with a single mismatch, the other with two) to the *chi* site of λ phage (consensus sequence = GCTGGTGG; not shown in figure). The tracts extend 5' the entire length of the gene (2.5 kb), with the other endpoint lying outside the class I gene. CON2 is donated by *H-2D^k* with an endpoint in exon 3, between positions 935 and 997. The tract extends 5' for the entire length of the *H-2K^k* gene (4.35 kb), and coconverts the *Arg4* gene for a total length in excess of 8 kb (see Fig. 1). The class I endpoint of this convertant also lies within a region of high sequence similarity between the class I genes, where 240 bp contain two separate heterozygosities. This region also included two sequence motifs, one with one mismatch and the other with two, resembling the consensus *chi* sequence (boxed; consensus sequence = gctgtgtg). CON3, donated by *H-2D^k*, has one endpoint ~350 bp into intron 3 from the exon 3 boundary between positions 4,365 and 4,379, a region of relatively high sequence heterology. The conversion tract extends 3' for the entire length of the gene (2.28 kb) and into the adjacent *Arg4* gene, involving a total of over 4 kb.

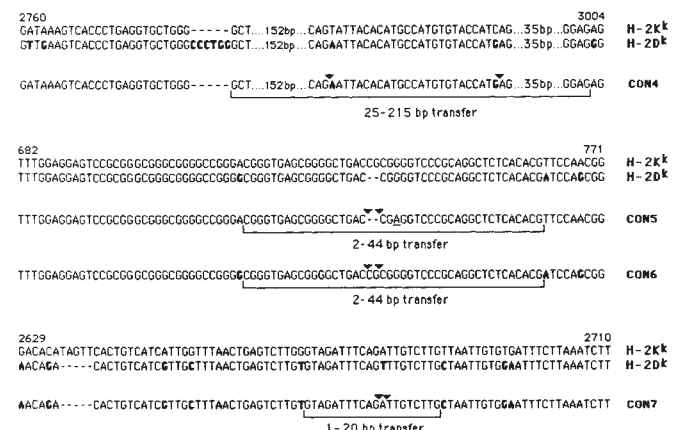


FIG. 3 Position and sequence of MHC class I microconversions in yeast. RNase protection revealed four microconversions (6% of unselected tetrads). Their positions are depicted in Fig. 2. Microconversion tracts are labelled 4, 5, 6 and 7 in the schematic, and include solid and dotted lines to indicate the minimum and maximum extent of conversion, respectively. The sequence of the microconversion tracts, labelled CON4, CON5, CON6 and CON7 are shown above with the parental sequences. CON4 is a donation of two *H-2D^k* specific nucleotides separated by 23 monomorphic nucleotides within exon 4. Flanking heterologies establish the maximum conversion tract length as 215 bp, between nucleotide positions 2,790 and 3,003, and the minimum as 25 bp. CON4 is in the same region as CON1, possibly associated with *chi*-like motifs (see above). CON5 is a conversion within intron 2 ranging from 2-44 bp, donated by *H-2D^k*. The maximum limits are at nucleotide positions 714 and 763. In addition, there is a point mutation (G to A; underlined) 3 bp away from the converted sequence. To confirm this, the parental *H-2D^k* and *H-2K^k* genes were cloned from two other spores in this tetrad and the corresponding regions sequenced; both carried the expected G. Although this mutation could have been conferred during plasmid rescue, we have not identified any other mutations in the class I genes in our yeast strains similarly manipulated. CON6 is a conversion of the same 2 bp as in CON5 but in the opposite direction, with *H-2D^k* as recipient, and no mutation is present. The 5' endpoint is adjacent to a GC-rich region that resembles the mouse I-E hotspot repeat¹⁶. CON7 has a conversion tract within intron 3 involving 1-20 bp donated by *H-2K^k* and located between positions 2,665 and 2,686, close to the start of exon 4, and is found in both *H-2D^k* spores.

H-2D^k as recipients, and class I germ-line mutations in which H-2K is the predominant recipient^{8,9} probably reflects the inherent bias in the method used to screen for such mutations in mice: events identified in the absence of selection for functionally distinct molecules do not reveal preferences for particular H-2 loci as donors or recipients^{27,28}.

Our ability to study recombination between divergent genes by placing them into yeast provides a system in which recombinants can be recovered and subjected to genetic and molecular

analysis in the absence of functional selection and phenotypic screening. By varying the degree of homology between polymorphic sequences, for example, we can now begin to dissect the level of heterology and other DNA sequence characteristics required to generate microconversions. Furthermore, the availability of yeast mutants affecting meiosis or meiotic recombination should facilitate the analysis of the specific enzymatic steps involved in the production of microconversions for any stretch of DNA regardless of the species. □

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Genetic linkage studies suggest that Alzheimer's disease is not a single homogeneous disorder

P. H. St George-Hyslop*, J. L. Haines*, L. A. Farrer†, R. Polinsky‡, C. Van Broeckhoven§, A. Goate||, D. R. Crapper McLachlan¶, H. Orr#, A. C. Bruni, S. Sorbi††, I. Rainero‡‡, J.-F. Foncin§§, D. Pollen|||, J.-M. Cantu¶¶, R. Tupler##, N. Voskresenskaya***, R. Mayeux†††, J. Growdon*, V. A. Fried*, R. H. Myers†, L. Nee‡, H. Backhovens§, J.-J. Martin§, M. Rossor||, M. J. Owen||, M. Mullan||, M. E. Percy¶, H. Karlinsky¶, S. Rich#, L. Heston#, M. Montesi**, M. Mortilla††, N. Nacmias††, J. F. Gusella*, J. A. Hardy|| & other members††† of the FAD Collaborative Study group**

ALZHEIMER'S disease, a fatal neurodegenerative disorder of unknown aetiology, is usually considered to be a single disorder because of the general uniformity of the disease phenotype^{1,2}. Two recent genetic linkage studies revealed co-segregation of familial Alzheimer disease with the D21S1/S11 and D21S16 loci on chromosome 21 (refs 3,4). But two other studies, one of predominantly multiplex kindreds with a late age-of-onset⁵, the other

of a cadre of kindreds with a unique Volga German ethnic origin⁶, found absence of linkage at least to D21S1/S11. So far it has not been possible to discern whether these conflicting reports reflect aetiological heterogeneity, differences in methods of pedigree selection, effects of confounding variables in the analysis (for example, diagnostic errors, assortative matings), or true non-replication. To resolve this issue, we have now examined the inheritance of five polymorphic DNA markers from the proximal long arm of chromosome 21 in a large unselected series of pedigrees with familial Alzheimer's disease. Our data suggest that Alzheimer's disease is not a single entity, but rather results from genetic defects on chromosome 21 and from other genetic or non-genetic factors.

Available members of 48 pedigrees (Table 1) were typed with five anonymous DNA markers (D21S16, D21S13, D21S52, D21S1 and D21S11) that constitute three independent loci whose physical and genetic relationships have been previously determined (Table 2)⁷⁻⁹. These data were then assessed using both maximum likelihood and affected pedigree member (APM) methods^{10,11}. The latter technique examines allele sharing among affected pedigree members only, and unlike the former, does not require specification of mode of transmission or the use of estimated parameters (for example, penetrance functions), thus eliminating these potential sources of inaccuracy. At the more informative D21S1/S11 and D21S13/S16 loci (% heterozygosity = 0.70 and 0.66 respectively) the maximum likelihood analyses provided pairwise lod scores exceeding the conventional value for acceptance of linkage (+3.00) (Table 2)¹⁰. The less

††† K. Abe*, L. Amaducci††, L. Bergamini††, M. Bruylant§, S. Cappa#, L. Connor*, G. De Winter§, D. Drachman||, R. G. Feldman†, M. Fracarro#, M. E. Franco¶¶, P. Frommelt§§§, S. I. Gavrilova***, G. Gei**, J. Gheuens§, A. Haynes||, J. Henry||, L. James||, M. F. James*, B. O'Donnell||, S. Piacentini††, L. Piness††, P. Roques||, C. Ruiz¶¶, J. Swearer||, R. E. Tanzi*, A. Vandenberghes, M. Vartanian***, G. Vaula††, P. C. Watkins|| & R. Williamson||

* Molecular Neurogenetics Lab., Depts Neurology and General Internal Medicine, Massachusetts General Hospital, 32 Fruit Street, Boston, MA 02114, USA

† Dept Neurology, Boston Univ. School of Medicine, 80 E. Concord Ave., Boston, MA 02118, USA

‡ Clinical Neuroscience Branch, NINDS, 9000 Rockville Pike, Bethesda, MD 20892, USA

§ Depts Neurogenetics and Neurology, Born Bunge Foundation, Univ. Antwerp, 1 Universiteitsplein, Antwerp, and Dept Neurology, Univ. Brussels, Laarbeeklaan 103/E, B-1090 Brussels, Belgium

¶ Depts Biochemistry and Neurology, St Mary's Hospital Medical School, Norfolk Place, London W2 1PG, UK

¶ Center for Research on Neurodegenerative Diseases, Tanz Neuroscience Institute, and Depts Medicine, Physiology, Psychiatry and Obstetrics, Faculty of Medicine, Univ. Toronto, King's College Circle, Toronto, Ontario, Canada, M5S 1A8

Depts Psychiatry and Laboratory Medicine, Univ. Minnesota Medical School, 420 Delaware St. Minneapolis, MN 55455, USA

** Centro Sud, Studio Multicentrico Italiano sulla Demenza, Via dei Campioni, 88046 Lamezia Terme CZ, Italy

†† Dept Neurology, Università di Firenze, viale Morgagni 85, Firenze, Italy

‡‡ Istituto di Clinica delle Malattie Nervose, Univ. Torino, via Cherasco 15, 10126 Torino, Italy

§§ Laboratoire de Neurohistologie, Ecole Pratique des Hautes Etudes and INSERM U106, Hôpital de la Salpêtrière, 75651 Paris 13, France

|| Dept Neurology, Univ. Massachusetts Medical School, Lake Ave. S., Worcester, MA 01605, USA

¶¶ Dipartimento di Patologia Umana ed Ereditaria, Università di Pavia, via Forlanini, 27100 Pavia, Italy, and Clinica Neurologica, Università di Brescia, Piazza Spedale Civili 1, 25125 Brescia, Italy

*** All Union Center for Mental Health Center, USSR Acad. Medical Sciences, Zagorodnoe sh. 2, Moscow 113-152, USSR

††† The Neurological Institute, Columbia Univ. College of Physicians and Surgeons, 710 W. 168th St, New York, NY 10032, USA

§§§ Kreis Krankenhaus Lemgo, Neurologische Abteilung, D492 Lemgo, FRG

|||| Life Technologies Inc., 8717 Governor Circle, Gaithersburg, MD 20887, USA

informative D21S52 locus (% heterozygosity = 0.58), located between these loci⁷, gave positive but nonsignificant scores. The APM analyses confirmed these results, showing significant association between familial Alzheimer's disease (FAD) and all three loci: D21S1/S11 ($T=3.30$, $P<0.001$); D21S52 ($T=3.07$, $P<0.002$); D21S13/S16 ($T=3.21$, $P<0.001$).

Although the two independent methods of analysis yielded significant overall evidence for linkage, not all pedigrees contributed positively to this result (Table 2). This admixture could arise if Alzheimer's disease (AD) comprises a heterogeneous group of disorders rather than a single entity. To investigate this possibility we applied the pre-divided sample test¹², which requires the families to be grouped according to an identifiable feature of the disorder. Currently, the most justifiable criterion is age-of-onset because prior work suggests both that this feature is consistent amongst affected members of the same family¹³⁻¹⁵ and that it may tentatively identify two phenotypic subgroups of AD, that is, presenile AD (onset ≤ 65 years of age) and senile dementia of the Alzheimer's type (SDAT; onset >65 years of age) which differ in several psychometric, neurochemical, genetic and epidemiological risk factors¹⁶⁻²².

When the pedigrees were subgrouped according to age-of-onset, the presenile pedigrees ($N=30$) displayed positive evidence of linkage to all three loci (maximum likelihood: see

Table 3; APM: D21S1/S11 ($T=4.08$, $P<0.001$), D21S52 ($T=3.78$, $P<0.001$), D21S13/S16 ($T=3.56$, $P<0.001$)). By contrast, the senile-onset pedigrees displayed minimal or negative evidence for linkage (maximum likelihood: exclusion of linkage to $\theta>0.06$ at all three loci; APM: weak association at D21S13/S16 ($T=2.42$, $P<0.01$) and no association at D21S1/S11 ($T=0.15$, $P=0.50$) or D21S52 ($T=0.88$, $P=0.45$)). The differences between presenile and senile onset pedigrees were statistically significant ($P<0.02$) (Table 3). To find further evidence for heterogeneity that might not be phenotypically discernible, we also applied the 'A' and 'B' tests of linkage heterogeneity to the ungrouped family-specific lod scores^{10,23}. Although neither of the latter tests provided statistical proof of heterogeneity ($P>0.10$), this does not negate the previous observations and more probably reflects lack of statistical power²³.

TABLE 1 Characteristics of linkage pedigrees

Pedigree number	Mean age of onset (yr)	Number of generations	Number of case with pathologic confirmation	Number of affected members sampled	Ethnic origin
1	52	8	7	7	Canadian
2	40	10	7	7	Italian
3	48	6	3	4	German
4	48	5	13	8	Russian Jewish
5	75	1	1	3	Russian Jewish
6	67	4	0	2	American
7	60	4	1	2	American
8	60	3	0	3	American
9	60	3	1	2	Canadian
10	48	5	7	3	Russian Jewish
11	61	2	1	4	American
12	74	3	0	3	Italian
13	60	3	1	3	Italian
14	62	2	0	2	American-Hispanic
15	71	2	1	2	Canadian
16	70	1	0	3	American
17	70	2	0	3	Russian
18	50	4	0	7	Mexican-Hispanic
19	45	4	0	5	Italian
20	79	3	1	3	Russian Jewish
21	35	6	11	10	Belgian
22	35	5	6	6	Belgian
23	79	1	0	3	British
24	89	2	0	5	British
25	74	2	0	3	British
26	56	3	1	6	British
27	80	1	0	4	British
28	80	2	0	2	British
29	57	2	1	1	British
30	60	2	1	2	British
31	78	1	0	3	British
32	43	2	1	2	British
33	62	1	0	1	British
34	65	1	1	1	British
35	84	1	1	3	British
36	65	1	0	2	British
37	76	2	0	1	British
38	64	1	0	2	British
39	66	1	0	0	British
40	71	1	0	2	British
41	42	5	0	1	British
42	54	2	0	1	British
43	45	3	7	1	American
44	62	3	3	1	American
45	71	3	0	4	American
46	45	3	3	3	American
47	65	3	2	3	American
48	65	2	1	1	American

Characteristics of pedigrees studied. The diagnosis of AD in living affected family members was achieved using NIA-NINCDS/ADRDA criteria^{25,26}. For deceased family members the diagnosis of AD was documented by the examination of medical and family records and, where available, by the neuropathologic examination of post-mortem brain tissue. A standardized record was used, and the molecular genetic data were not revealed to the researchers involved in diagnosis. Preliminary linkage data on pedigrees 1-4, 26, 29, 30, 32, 33 have been previously published^{3,4}, but have been substantially updated and expanded during the intervening two to three years.

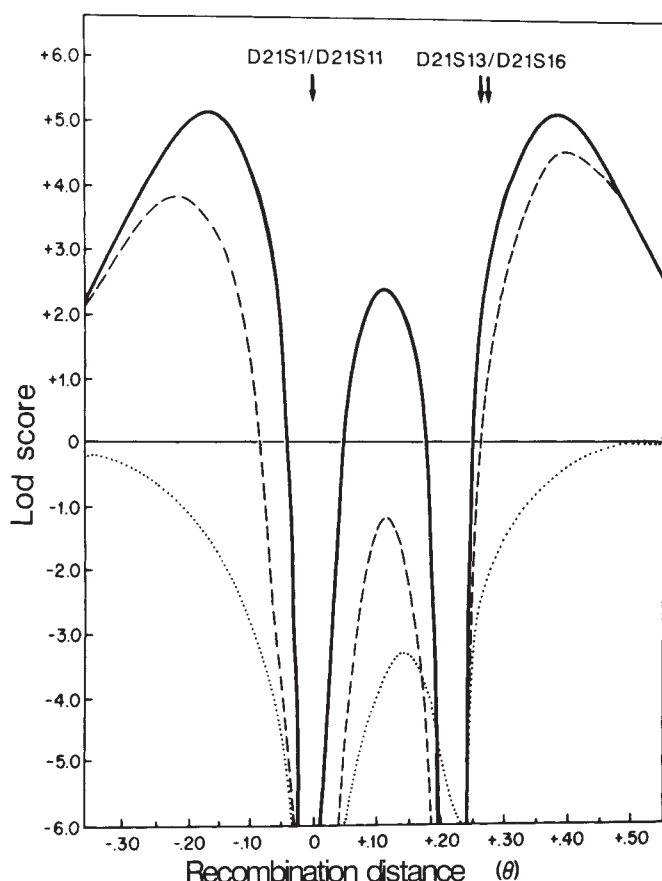


FIG. 1 Multipoint linkage analyses were performed using the LINKAGE (version 4.9)²⁷ programs to calculate the relative likelihoods ($\log_{10}$ likelihood differences) for placement of the disease gene at various theoretical locations around the fixed map positions of D21S1/S11 (telomeric) and D21S13/S16 (centromeric). Owing to computational complexity, data from the internal and less informative D21S52 locus could not be included in the analysis. Gene frequency, mutation rate and penetrance functions were the same as those used for the two-point analyses. Multipoint analyses were computed for the entire data set (—), the presenile onset group (---), and for the senile onset group (····).

TABLE 2 Two-point lod scores for chromosome 21 markers

Pedigree number	D21S13/D21S16						D21S52						D21S1/D21S11					
	lod score at recombination fraction (θ)						lod score at recombination fraction (θ)						lod score at recombination fraction (θ)					
	0.00	0.05	0.10	0.20	0.30	0.40	0.00	0.05	0.10	0.20	0.30	0.40	0.00	0.05	0.10	0.20	0.30	0.40
1	-∞	-1.59	-0.87	-0.24	-0.05	-0.02	-∞	0.16	0.34	0.27	0.10	0.00	-∞	0.73	0.74	0.51	0.24	0.06
2	-∞	2.19	2.17	1.72	1.12	0.48	-∞	1.55	2.30	2.44	1.86	0.93	-∞	0.94	0.92	0.66	0.39	0.12
3	0.33	0.22	0.12	0.00	-0.04	-0.02	-3.22	-0.76	-0.48	-0.22	-0.10	-0.03	-∞	-0.29	0.00	0.14	0.10	0.03
4	-∞	-2.34	-1.57	-0.86	-0.59	-0.32	-5.79	-0.52	-0.01	0.24	0.18	0.07	-∞	0.47	0.64	0.62	0.44	0.23
5	-0.24	-0.19	-0.15	-0.08	-0.04	-0.01	-1.56	-0.93	-0.69	-0.41	-0.23	-0.10	-0.13	-0.07	-0.03	0.00	0.01	0.00
6	-∞	-0.67	-0.40	-0.16	-0.06	-0.01	0.04	0.02	0.01	-0.01	-0.02	-0.02	-0.32	-0.23	-0.17	-0.09	-0.04	-0.01
7	0.08	0.07	0.06	0.04	0.02	0.00	-0.14	-0.14	-0.14	-0.11	-0.07	-0.03	0.16	0.14	0.11	0.06	0.03	0.01
8	0.06	0.04	0.02	-0.01	-0.01	-0.01	-0.09	-0.09	-0.08	-0.06	-0.03	-0.01	0.15	0.13	0.10	0.06	0.03	0.01
9	0.10	0.09	0.07	0.05	0.02	0.01	0.05	0.03	0.01	-0.02	-0.02	-0.02	0.27	0.23	0.19	0.11	0.05	0.01
10	-0.67	-0.49	-0.36	-0.19	-0.09	-0.03	0.48	0.34	0.22	0.05	-0.02	-0.04	0.72	0.66	0.60	0.46	0.31	0.15
11	-2.28	-0.42	-0.19	-0.03	0.01	0.01	-2.26	-0.48	-0.25	-0.07	0.01	0.00	-0.28	-0.06	0.03	0.08	0.06	0.02
12	-0.96	-0.51	-0.31	-0.13	-0.05	-0.01	-0.72	-0.41	-0.26	-0.11	-0.04	-0.01	-2.71	-0.63	-0.37	-0.15	-0.05	-0.01
13	0.02	0.01	0.01	0.01	0.00	0.00	-3.10	-0.77	-0.46	-0.19	-0.07	-0.01	0.25	0.20	0.15	0.09	0.04	0.02
14	-0.03	0.01	0.03	0.04	0.03	0.01	0.09	0.15	0.18	0.18	0.14	0.08	0.50	0.42	0.34	0.21	0.10	0.03
15	0.14	0.12	0.09	0.06	0.03	0.01	-0.48	-0.34	-0.24	-0.11	-0.04	0.00	0.03	0.02	0.01	0.00	0.00	0.00
16	-0.17	-0.13	-0.09	-0.05	-0.02	0.00	0.44	0.38	0.33	0.23	0.14	0.06	-0.16	-0.12	-0.09	-0.05	-0.02	-0.01
17	0.53	0.46	0.39	0.25	0.13	0.04	-0.26	-0.22	-0.18	-0.10	-0.05	-0.01	-0.21	-0.18	-0.15	-0.09	-0.04	-0.01
18	-∞	-0.43	-0.05	0.22	0.22	0.11	-∞	-0.80	-0.53	-0.26	-0.11	-0.02	-∞	-0.96	-0.52	-0.21	-0.11	-0.05
19	-∞	-0.84	-0.36	-0.02	0.05	0.04	0.29	0.28	0.26	0.21	0.14	0.07	-∞	-0.52	-0.27	-0.08	-0.03	-0.01
20	-1.11	-0.63	-0.41	-0.18	-0.07	-0.02	0.06	0.04	0.03	0.01	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00
21	-∞	0.38	0.77	0.84	0.57	0.19	-∞	-0.12	0.35	0.50	0.33	0.06	-∞	-1.88	-0.85	-0.12	0.02	-0.03
22	0.63	0.64	0.60	0.44	0.26	0.10	-0.77	-0.74	-0.71	-0.58	-0.42	-0.25	-∞	-0.65	-0.21	0.14	0.18	0.09
23	-0.20	-0.15	-0.12	-0.06	-0.03	-0.01	-0.22	-0.17	-0.13	-0.07	-0.03	-0.01	0.03	0.02	0.02	0.01	0.01	0.00
24	-0.22	-0.21	-0.19	-0.12	-0.06	-0.01	-1.07	-1.05	-0.95	-0.61	-0.32	-0.12	-2.07	-1.37	-0.88	-0.39	-0.15	-0.04
25	-0.29	-0.25	-0.21	-0.12	-0.05	-0.01	-0.11	-0.08	-0.05	-0.02	0.00	0.01	-3.21	-0.99	-0.65	-0.30	-0.12	-0.03
26	2.20	1.99	1.77	1.30	0.80	0.29	-1.69	-0.39	-0.16	0.03	0.08	0.06	2.05	1.84	1.62	1.16	0.68	0.23
27	0.40	0.34	0.28	0.17	0.08	0.02	-1.99	-0.68	-0.43	-0.20	-0.09	-0.03	-5.05	-1.43	-0.88	-0.39	-0.15	-0.04
28	0.08	0.07	0.06	0.03	0.02	0.00	-1.06	-0.94	-0.74	-0.41	-0.20	-0.07	0.43	0.37	0.31	0.20	0.10	0.03
29	-0.02	-0.02	-0.02	-0.01	0.00	0.00	0.39	0.31	0.23	0.07	-0.06	-0.09	0.51	0.44	0.37	0.23	0.11	0.03
30	0.39	0.34	0.29	0.18	0.09	0.02	0.82	0.75	0.68	0.52	0.35	0.17	-0.09	-0.06	-0.04	-0.01	-0.01	0.00
31	-2.25	-0.44	-0.21	-0.06	-0.01	0.00	-0.43	-0.38	-0.34	-0.26	-0.18	-0.09	0.10	0.08	0.07	0.04	0.02	0.00
32	-0.12	-0.10	-0.08	-0.04	-0.02	0.00	-0.23	-0.19	-0.16	-0.09	-0.04	-0.01	-3.87	-0.65	-0.38	-0.15	-0.05	-0.01
33	0.39	0.39	0.36	0.26	0.15	0.04	-0.01	0.00	0.00	0.00	0.00	0.00	0.26	0.28	0.27	0.20	0.11	0.03
34	-0.01	-0.01	-0.01	0.00	0.00	0.00	0.03	0.03	0.02	0.02	0.01	0.01	-0.16	-0.12	-0.09	-0.05	-0.02	-0.01
35	-0.11	-0.09	-0.07	-0.04	-0.02	0.00	-1.52	-0.38	-0.15	0.02	0.06	0.05	0.08	0.07	0.06	0.03	0.02	0.00
36	0.02	0.02	0.01	0.01	0.00	0.00	0.08	0.08	0.07	0.06	0.04	0.02	0.00	0.00	0.00	0.00	0.00	0.00
37	0.27	0.23	0.20	0.12	0.06	0.01	-0.11	-0.09	-0.08	-0.05	-0.03	-0.01	-0.06	-0.05	-0.04	-0.02	-0.01	0.00
38	-0.04	-0.03	-0.02	-0.01	0.00	0.00	-0.05	-0.06	-0.06	-0.07	-0.06	-0.03	0.00	0.00	0.00	0.00	0.00	0.00
39	-0.02	-0.01	-0.01	-0.01	0.00	0.00	-0.01	-0.01	-0.01	-0.01	0.00	0.00	-0.01	-0.01	-0.01	-0.01	0.00	0.00
40	0.03	0.03	0.02	0.01	0.01	0.00	0.08	0.04	0.00	-0.06	-0.08	-0.06	0.00	0.00	0.00	0.00	0.00	0.00
41	0.06	0.05	0.04	0.02	0.01	0.00	-0.30	-0.21	-0.15	-0.07	-0.03	0.00	-0.04	-0.04	-0.03	-0.02	-0.01	0.00
42	-0.04	-0.03	-0.02	-0.01	0.00	0.00	-0.18	-0.15	-0.12	-0.08	-0.05	-0.02	0.34	0.29	0.24	0.15	0.07	0.02
43	-1.39	-0.55	-0.32	-0.13	-0.05	-0.01	-0.55	-0.41	-0.31	-0.18	-0.10	-0.04	-0.27	-0.18	-0.12	-0.06	-0.02	-0.01
44	0.00	0.00	0.00	0.00	0.00	0.00	-0.14	-0.12	-0.11	-0.08	-0.05	-0.02	0.00	0.00	0.00	0.00	0.00	0.00
45	-0.32	-0.03	0.07	0.12	0.08	0.02	-2.39	-0.67	-0.40	-0.16	-0.06	-0.01	-3.29	-0.17	0.03	0.13	0.10	0.03
46	-2.69	-0.70	-0.39	-0.12	-0.02	0.00	-1.05	-0.79	-0.44	-0.03	0.12	0.11	-1.32	-0.79	-0.48	-0.19	-0.06	0.00
47	0.14	0.12	0.09	0.05	0.02	0.01	-0.62	-0.52	-0.42	-0.26	-0.13	-0.05	0.65	0.57	0.49	0.32	0.17	0.05
48	0.03	0.02	0.02	0.01	0.01	0.00	-0.35	-0.27	-0.22	-0.13	-0.07	-0.03	-0.01	-0.01	-0.01	-0.00	0.00	0.00
Total	-∞	-3.04	1.11	3.26	2.48	0.91	-∞	-9.72	-4.40	-0.24	0.78	0.45	-∞	-3.54	1.04	3.22	2.47	0.92
Peak lod score ($\hat{z}$ at $\hat{\theta}$)	3.27 at 0.21						0.78 at 0.29						3.23 at 0.21					

The genotype of available pedigree members was ascertained as previously for the D21S1/S11 (pPW228C and pPW236B), D21S52 (pPW511-1H and pPW511-12P) and D21S13/S16 (pGSM21 and pGSE9) loci^{3,4}. Family-specific 'pairwise' $\log_{10}$ likelihood differences (lod scores - z) at various recombination fractions (θ) and APM scores were calculated using LIPED (version 3.0)²⁷ or LINKAGE (version 4.9)²⁸ and the two-point or multi-point APM programs¹¹ respectively. Members of the marker pairs D21S1/D21S11 and D21S13/D21S16 are tightly linked genetically and physically⁷⁻⁹. The data contributed by each of these marker pairs were considered as a single extended locus (for D21S1/S11, $\theta=0.00$; for D21S13/S16 a conservative $\theta=0.02$ was assumed). Allele or haplotype frequencies were as published⁷, or deduced from 75-150 chromosomes of unrelated spouses. The initial analysis assumed: autosomal dominant inheritance; an FAD allele frequency of 0.001; a new mutation rate of 0.00; and an age-of-onset correction for asymptomatic individuals^{15,29}. The analyses were repeated varying FAD allele frequency (0.05-0.00001) and mutation rate (0.00-0.001) without significantly altering the results. When data from only affected members was used the lod scores remained positive but reduced (D21S1/S11, $\hat{z}=1.33$ at $\hat{\theta}=0.24$; D21S52 $\hat{z}=0.02$ at $\hat{\theta}=0.44$; D21S13/S16 $\hat{z}=1.23$ at $\hat{\theta}=0.24$) because a substantial portion of the available genetic data is removed from the analysis.

To determine whether the two previous reports^{5,6} showing negative overall scores for chromosome 21 markers are consistent with our conclusion of heterogeneity, we combined our data at D21S1/S11 and D21S13/S16 with that published for D21S1/S11 and D21S16 in these reports (neither report tested D21S52 or D21S13), applied the same *a priori* age-of-onset criterion, and repeated the pre-divided sample test. This re-analysis remained nonsignificant at D21S13/S16 because D21S16 was minimally informative in the latter reports. However, consistent with our hypothesis, heterogeneity was clearly demonstrable at D21S1/S11 regardless of whether the Volga German pedigrees were considered as a distinct subgroup²⁰ ($\chi^2=16.1$, $P<0.005$, d.f.=2) or were included in the presenile group ($\chi^2=8.75$, $P<0.005$, d.f.=1).

To extend these observations we also performed multilocus analyses using both maximum likelihood and APM methods. Significant evidence for linkage was again observed in the current data set (maximum likelihood: $\hat{z}=+4.50$; APM: $T=4.48$, $P<0.0001$) (Fig. 1). However, as with the pairwise analyses, no positive evidence for linkage was observed in the senile onset kindreds using either the maximum likelihood (Fig. 1) or APM methods ($T=0.50$, $P=0.34$). By contrast, significant evidence for linkage was again provided by the presenile onset pedigrees (maximum likelihood: $\hat{z}=5.03$ (Fig. 1); APM: $T=4.86$, $P<0.0001$).

Figure 1 reveals that the genetic defect in the presenile onset pedigrees cannot be definitively placed relative to the current markers. This ambiguity may reflect the effect of 'false' recomb-

TABLE 3 Cumulative lod scores for pedigrees divided according to age-of-onset

Locus	Age of onset (yr)	Cumulative lod score at recombination fraction (θ)						Exclusion of linkage ($Z < -2.0$)	Peak lod score ($\hat{Z}$ at $\hat{\theta}$)	Significance	
		0.00	0.05	0.10	0.20	0.30	0.40			χ^2	P
D21S13/D21S16	≤ 65	$-\infty$	-0.97	2.17	3.51	2.49	0.90	—	3.60 at 0.17	1.34	>0.30
	>65	$-\infty$	-2.07	-1.06	-0.25	-0.02	0.02	$\theta = 0.06$	0.02 at 0.40		
D21S52	≤ 65	$-\infty$	-3.86	-0.13	2.09	1.93	0.85	$\theta = 0.06$	2.22 at 0.24	6.45	<0.02
	>65	-11.3	-5.86	-4.27	-2.33	-1.15	-0.40	$\theta = 0.22$	—		
D21S1/D21S11	≤ 65	$-\infty$	1.14	3.81	4.28	2.82	1.00	—	4.50 at 0.17	5.89	<0.02
	>65	-16.5	-4.68	-2.77	-1.06	-0.35	-0.08	$\theta = 0.13$	—		

Cumulative two-point lod scores at D21S1/S11, D21S52 and D21S13/S16 for the presenile-onset (family-specific mean-age-of-onset ≤ 65 years) and the senile-onset subgroups (family-specific mean-age-of-onset > 65 years). The pre-divided sample test was used to assess the significance of linkage heterogeneity between the two groups. Similar results were observed if age-of-onset 60 years or 70 years were used ($\hat{Z} > +3.00$ at D21S1/S11 and D21S13/S16 in early onset group; strong evidence against linkage in the late-onset group).

nation events resulting from the inclusion of a few nonallelic pedigrees within the presenile group (that is, age-of-onset *per se* may be only an imperfect discriminator of subtypes of this disease). However, the same effect would result from 'false' recombinants contributed by diagnostic errors, by undiscovered nonpaternity, and by the occurrence of new mutations or sporadic AD phenocopies in non-FAD gene carriers²⁴. Finally, we cannot exclude the possibility that structural rearrangements of chromosome 21 may occur in FAD, or that the FAD defect in individual families may exist at slightly different locations on chromosome 21.

Our observations indicate that there is more than one cause of AD. Thus, in some instances, AD is caused by a genetic defect on chromosome 21. In other instances, the disease results from other mono- or polygenic genetic defects, from nongenetic agents, or from mixed genetic and environmental factors. These observations have important implications for other areas of research into this distressing disorder. $\square$

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Activation of muscle genes without myogenesis by ectopic expression of MyoD in frog embryo cells

N. D. Hopwood & J. B. Gurdon

Cancer Research Campaign Molecular Embryology Group,
Department of Zoology, Downing Street, Cambridge CB2 3EJ, UK

THE sequence-specific DNA-binding protein, MyoD^{1,2}, can activate muscle-specific gene expression in some cells in culture^{1,3,4}. *Xenopus MyoD* (*XMyoD*) transcription is activated as a consequence of mesoderm induction in the early myotomes⁵, from which the axial musculature develops. *XMyoD* RNA accumulates about two hours before muscle-specific actin transcripts first appear⁵, and so is expressed at the right time and in the right place to play a part in activating muscle-specific gene expression in normal development (but see ref. 6). To test this idea, we have expressed *XMyoD* ectopically in early *Xenopus* embryos. We find that injection of *XMyoD* RNA can strongly activate muscle genes in embryo cells normally destined to form ectoderm. Nevertheless, these cells fail to differentiate as muscle, suggesting that additional factors are required for complete and stable myogenesis.

Microinjection of synthetic RNA into early *Xenopus* embryos has been successfully used to create uniformly high levels of several messenger RNAs that are normally found only in part of the embryo⁷⁻¹⁰. We have used this procedure with *XMyoD*. To maximize the efficiency of translation of the injected mRNA in embryos, we constructed a hybrid complementary DNA (Fig. 1a) containing the *XMyoD* coding sequence flanked by the 5' and 3' untranslated regions from a *Xenopus* β -globin gene¹¹. A control RNA was made by mutating a G residue to a C, changing alanine 114 to a proline (Fig. 1a). This would be expected to disrupt the first helix of the helix-loop-helix dimerization domain of the *XMyoD* protein¹², preventing dimerization and thus DNA binding. A similar mutation in mouse *MyoD* made it unable to dimerize or to convert C3H10T $\frac{1}{2}$ mouse embryo fibroblasts to myoblasts¹³. RNAs made from this *XMyoD114P* mutant and the wild-type clone are translated with the same efficiency *in vitro* (Fig. 1b).

We have tested the effect of injection of *XMyoD* RNA in animal cap cells. The mesoderm, including muscle, develops from equatorial animal hemisphere cells of the blastula in response to induction by underlying vegetal cells^{14,15}. The animal

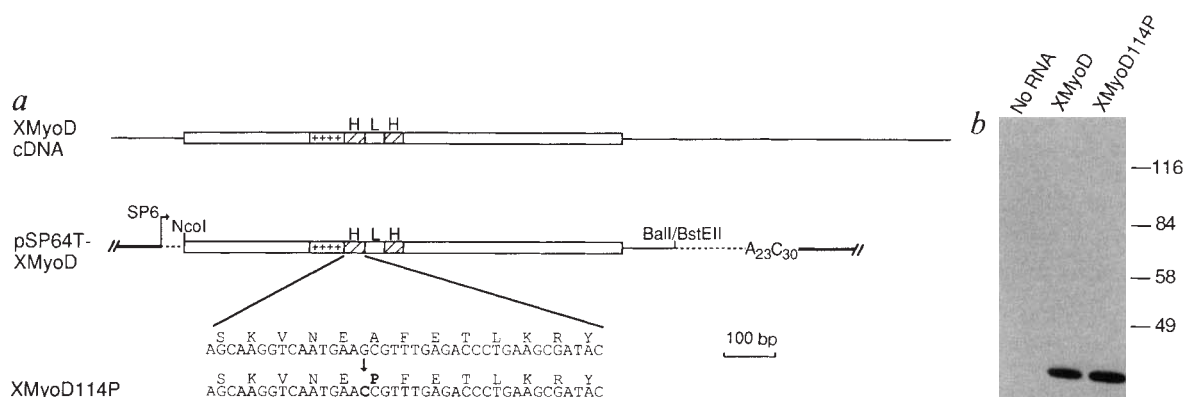


FIG. 1 *XMyoD* transcription templates and *in vitro* translation. *a*, *Xenopus* *MyoD* cDNA (XMyoD2-24, ref. 5), and derived transcription clones designed to promote mRNA translation in embryos, and to provide a negative control for the effects of *XMyoD* RNA injection. HLH, helix-loop-helix domain; + + + +, basic region; dotted line, β -globin 5' and 3' UTRs (untranslated regions); $A_{23}C_{30}$, poly(dA)-poly(dC) tracts. *b*, Translation of RNA transcribed from the pSP64T-XMyoD and -XMyoD114P clones in a rabbit reticulocyte lysate. Autoradiograph of ^{35}S -labelled protein. Like mouse *MyoD*²⁴ and chicken *CMD1*²⁰, the *Xenopus* protein migrates more slowly during SDS-PAGE than would be predicted from its sequence (at relative molecular mass 42,000–43,000, *M*, 42–43K, rather than 32K).

METHODS. Plasmid pSP64T-XMyoD was constructed from XMyoD2-24 by creating a *Nco*I site around the first ATG. The *Nco*I–*Bal*I fragment containing the coding sequence and 100 base pairs (bp) of 3' UTR was then subcloned into pSP64-X β m (ref. 11) to replace the *Xenopus* β -globin coding sequence, which had been removed by digestion with *Nco*I and *Bst*II. A point mutant derivative of this clone (pSP64T-XMyoD114P) was made by oligonucleotide-directed *in vitro* mutagenesis using a Version 2 Amersham kit; DNA sequen-

cing confirmed that there were no other changes in the coding region. Trace-labelled RNA was produced as described²⁵ using SP6 RNA polymerase and templates linearized with *Xba*I, capped by inclusion of $m^7\text{GpppG}$ (New England Biolabs) in the reaction, and unincorporated nucleotides removed by three rounds of ethanol precipitation. *In vitro* translation²⁶ used reticulocyte lysate (R. T. Hunt, Department of Biochemistry, University of Cambridge, UK).

cap, comprising the cells furthest from the equator, normally escapes this induction and forms only ectodermal derivatives such as epidermis, but it can be induced to make muscle experimentally if it is combined with vegetal cells^{14,15}. We injected *XMyoD* or *XMyoD114P* RNA into two-cell embryos, separated the animal caps at the late blastula stage (stage 9, ref. 16), and cultured them until control whole embryos became late neurulae. The animal caps were then assayed by northern blotting for cardiac actin transcripts¹⁷, which at this stage are normally present only in the myotomes. This showed that the cardiac actin gene was activated in the animal caps from *XMyoD*-injected embryos, but not in controls injected with mutant RNA (Fig. 2a). *XMyoD* also activated the skeletal actin gene¹⁷ (data not shown). *XMyoD* can thus bypass mesoderm-inducing factors and activate muscle-specific gene expression in animal cap cells.

It is important to compare the effectiveness of ectopic muscle gene activation by *XMyoD* with muscle gene activity in normally developing muscle cells at the same stage. At the early neurula stage, the cardiac actin gene has been activated in animal caps from *XMyoD*-injected embryos to an extent similar to its normal activation in the myotomes (Table 1). But this is achieved by a roughly 10-fold greater concentration of *XMyoD* RNA in animal caps than exists in the myotomes of uninjected embryos. This could be because the injected RNA is less well translated than endogenous RNA, or because animal cap cells are less responsive than future muscle cells, as would be the case if other specific factors were involved in the normal activation of the cardiac actin gene in the myotomes. Although we have not measured *XMyoD* protein levels, the achievement of a normal degree of activation of the cardiac actin gene implies that biologically active *XMyoD* protein is present in cell nuclei. In contrast to the continuous rapid accumulation of cardiac actin RNA in the myotomes, transcription of the cardiac actin gene in *XMyoD*-injected animal caps fails to be sustained after its

initial activation, possibly as a consequence of progressive degradation of the injected RNA (Table 1).

Experiments in cultured cells have shown that *MyoD* can autoactivate, and it has been suggested that this could be responsible for the maintenance of muscle differentiation^{18–20}. We therefore asked whether autoactivation occurs in the *XMyoD*-injected animal caps. A probe specific for endogenous *XMyoD* transcripts showed that the endogenous *XMyoD* gene(s) is activated in *XMyoD*- but not in *XMyoD114P*-injected animal caps (Fig. 2b). Autoactivation can thus occur in early embryonic

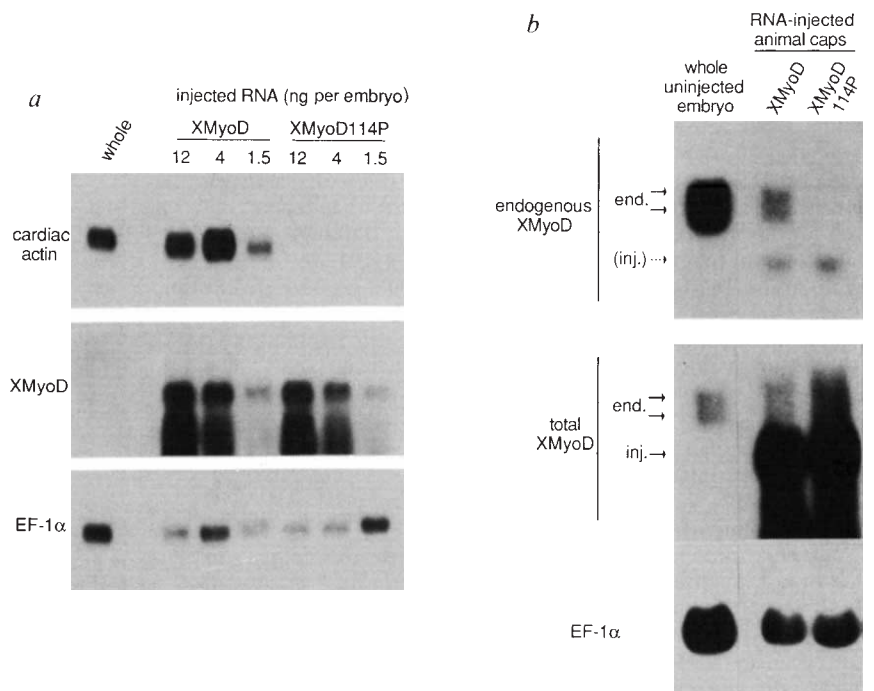
TABLE 1 Effectiveness of ectopic muscle gene activation by *XMyoD*

	Transcript number* $\times 10^{-5}$			
	Early neurula (stage 13)		Late neurula (stage 18)	
	<i>XMyoD</i>	Cardiac actin	<i>XMyoD</i>	Cardiac actin
Myotomes of uninjected embryos	110	100	110	350
<i>XMyoD</i> -injected animal caps	inj.† 1,200 $\pm$ 400 end.† 2.6 $\pm$ 0.6	71 $\pm$ 27	inj. 390 $\pm$ 80 end. 2.5 $\pm$ 0.5	54 $\pm$ 17

* Values are derived from densitometry of appropriate autoradiographic exposures of northern blots like the ones shown in Fig. 2. They are given for equivalent amounts of uninjected myotomes (estimated to have a mass $\frac{1}{10}$ that of a whole embryo) and animal cap tissue from embryos injected with *XMyoD* RNA (5 ng). The values are expressed as numbers of transcripts per embryo equivalent of EF-1 α (ref. 23), based on transcript number determinations for cardiac actin (2.5×10^8 transcripts per embryo at stage 26; T. J. Mohun, personal communication) and *XMyoD* (ref. 5). Means $\pm$ s.e.m.; *n* = 3.

† inj., injected RNA (numbers of full-length transcripts only); end., transcripts from endogenous gene due to autoactivation by injected *XMyoD* RNA.

FIG. 2 *XMyoD* activates muscle gene expression in animal cap cells. Northern blots of RNA extracted from animal caps dissected at the late blastula stage (st9) from embryos injected with either *XMyoD* or *XMyoD114P* mutant RNA and cultured until control whole embryos reached the late neurula stage (st18). *a*, Cardiac actin RNA is present in *XMyoD*-injected, but not in *XMyoD114P*-injected, animal caps. Re-probing for *XMyoD* showed the animal cap samples to contain roughly comparable amounts of surviving *XMyoD* or *XMyoD114P* RNA. The degradation products of the injected RNA are seen as a smear below the full-length band. The *XMyoD* transcripts in the whole uninjected embryo sample (1 µg of total stage-18 RNA) included for comparison were visible only on a longer autoradiographic exposure. Re-probing for *EF-1α* RNA²³ show the relative amounts of total RNA in the animal cap samples. *b*, Autoactivation of the *XMyoD* gene. *XMyoD*, but not *XMyoD114P*, RNA activates endogenous *XMyoD* transcription. The top panel shows hybridization of a probe specific for endogenous *XMyoD* transcripts. The injected RNAs (dotted arrow) are seen faintly on this autoradiograph because of the radionucleotides incorporated into them; the intensity of these bands thus does not reflect the abundance of injected relative to endogenous transcripts. This is shown in the middle panel by re-probing the blot with a probe made from the entire *XMyoD*2–24 cDNA⁵, which detects both injected and endogenous transcripts. Note the very large amount of injected RNA relative to autoactivated transcripts in the animal cap samples. **METHODS.** *In vitro* synthesized RNA (see Fig. 1 legend) was injected into both cells of two-cell *X. laevis* embryos, which were cultured as described²⁷. RNA from two (*a*) or eight (*b*) animal caps was loaded in each lane. Northern blots for *XMyoD* and *EF-1α* were as described⁵. The single-stranded DNA probe for endogenous *XMyoD* transcripts was prepared from a *PvuII*–*EcoRI*



subclone comprising the terminal 284 bases of the 3' untranslated region of the *XMyoD*2–24 cDNA⁵ (sequence not present in the injected RNA) inserted between the *SmaI* and *EcoRI* sites of M13mp19. Cardiac actin RNA was detected using a RNA probe (pSP21S, provided by T.J. Mohun) by hybridization in 50% formamide, 10% dextran sulphate, 5 × SSPE buffer, 1% SDS, 200 µg ml⁻¹ sheared, denatured salmon sperm DNA at 65 °C, followed by washing to 0.2 × SSPE, 0.1% SDS at 65 °C.

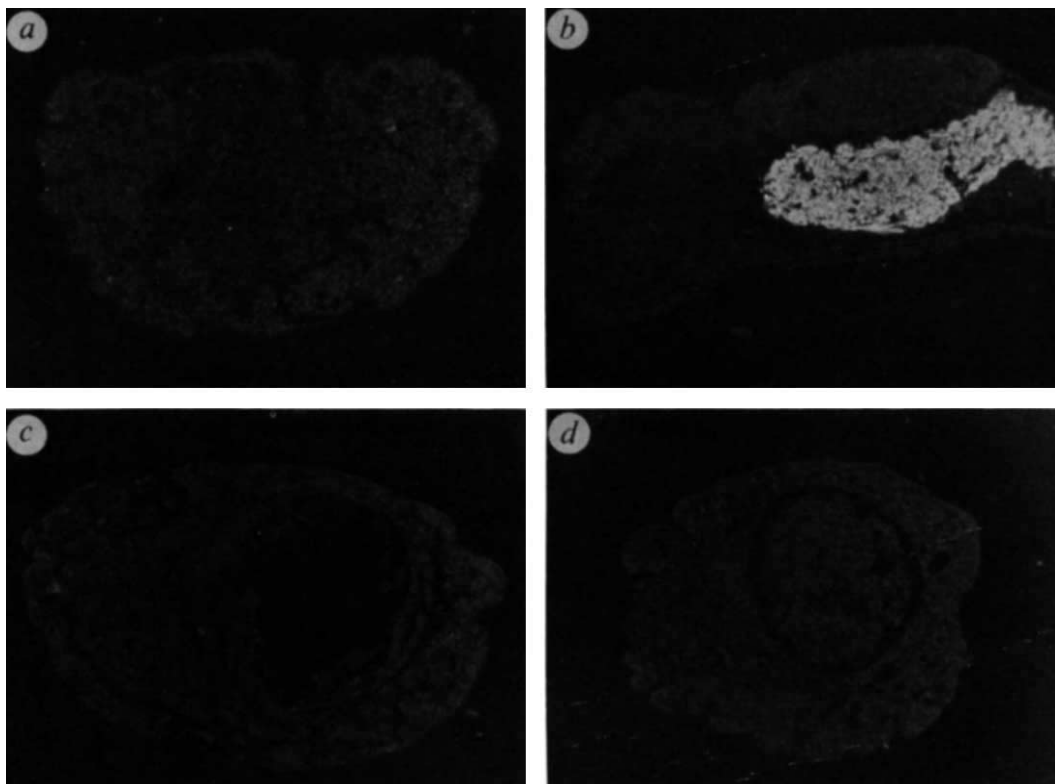


FIG. 3 Lack of muscle differentiation in *XMyoD*-injected animal caps. Sections of animal caps that were dissected from late blastulae, cultured as fused pairs or in combination with inducing vegetal tissue until sibling embryos became stage-35 tadpoles, and then stained with the 12/101 anti-muscle antibody²¹. *a*, Uninjected, *b*, uninjected animal cap conjugated with inducing vegetal tissue; *c*, *XMyoD*-injected, *d*, *XMyoD114P*-injected. Only injected animal caps show labelling above background. **METHODS.** *XMyoD*-injected animal caps were from batches that were shown by northern blotting to have activated the cardiac actin gene. Methanol-fixed paraffin sections (10 µm) were stained with antibody as described²¹.

cells, although the number of endogenous *XMyoD* transcripts is much less than that normally found in the myotomes (Table 1).

Do animal caps isolated from *XMyoD*-injected embryos show other signs of muscle differentiation? The *XMyoD*-injected animal caps are histologically indistinguishable from uninjected or *XMyoD114P*-injected controls (Fig. 3a, c, d). By contrast, uninjected animal caps induced by vegetal tissue elongate and contain large blocks of muscle (Fig. 3b). We obtained intense labelling of this muscle tissue using the 12/101 anti-muscle antibody²¹, but saw no labelling above background of the *XMyoD*-injected animal cap cells (Fig. 3). We conclude that no differentiated muscle is formed by *XMyoD*-injected animal caps. Thus, animal cap cells that contain as much cardiac actin RNA as normal myotomal cells do not express the full myogenic programme, but continue to differentiate as epidermis. Whole *XMyoD*-injected embryos also develop relatively normally, becoming tadpoles with substantially normal external and internal structures, including a variety of differentiated cell types (data not shown).

We have shown that *XMyoD* can activate a muscle gene to its normal level in animal cap cells. *MyoD* can bind to sites in the promoters of muscle genes², and we note that, in addition to a CArG sequence that is essential for transcription²², there are potential *XMyoD*-binding sites² located further upstream in the *Xenopus* cardiac actin promoter (M. V. Taylor, N.D.H. and T. J. Mohun, unpublished data). The lack of muscle differentiation in *XMyoD*-injected animal caps could be caused by a failure to maintain a sufficiently high concentration of *XMyoD* protein. Alternatively, as enough *XMyoD* has been supplied to activate the cardiac actin gene to its normal level, it may be that muscle did not differentiate because other

myogenic factors, not themselves activated by *XMyoD*, are required to divert these embryonic cells from their normal pathway of differentiation. □

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Enhancement of chilling tolerance of a cyanobacterium by genetic manipulation of fatty acid desaturation

Hajime Wada, Zoltan Gombos & Norio Murata*

National Institute for Basic Biology, Myodaiji, Okazaki 444, Japan

THE sensitivity (or tolerance) of plants to chilling determines their choice of natural habitat and also limits the worldwide production of crops. Although the molecular mechanism for chilling sensitivity has long been debated, no definitive conclusion has so far been reached about its nature. A probable hypothesis^{1,2}, however, is that chilling injury is initiated by phase transition of lipids of cellular membranes, as demonstrated for cyanobacteria, which serve as a model system for the plant cells^{3,4}. Because the phase transition temperature depends on the degree of unsaturation of fatty acids of the membrane lipids⁵, it is predicted that the chilling tolerance of plants can be altered by genetically manipulating fatty-acid desaturation by introducing double bonds into fatty acids of membrane lipids. Here we report the cloning of a gene for the plant-type desaturation (termed *desA*). The introduction of this gene from a chilling-resistant cyanobacterium, *Synechocystis* PCC6803, into a chilling-sensitive cyanobacterium, *Anacystis nidulans*, increases the tolerance of the recipient to low temperature.

A mutant in fatty-acid desaturation of membrane lipids of the transformable cyanobacterium, *Synechocystis* PCC6803, was isolated as described previously⁶. This mutant, termed Fad12,

is defective in the activity of desaturation that introduces a second double bond at the Δ^{12} position of the C₁₈ fatty acids of membrane lipids⁶. It grows much slower at low temperatures (such as 22 °C) than the wild type⁶.

TABLE 1 Composition of major fatty acids of total membrane lipids in various strains of *Synechocystis* PCC6803

Strain	Fatty acid (mol %)					
	16:0	18:0	18:1 (9)	18:2 (6,9)	18:2 (9,12)	18:3 (6,9,12)
Wild type	58	1	8	t	12	17
Mutant (Fad12)	59	1	32	5	t	t
Transformant of Fad12 with <i>desA</i> (Bluescript/1.5 kbp)	59	2	9	1	12	14
Transformant of Fad12 with <i>desA</i> (pTZ19R/8 kbp)	59	1	8	t	12	15
Transformant of wild type with <i>desA::Km^r</i> (Bluescript/1.5 kbp::Km ^r)	60	1	32	5	t	t

t, trace amount (<0.4%). Wild type, mutant (Fad12), and transformants of Fad12 with *desA* were grown photoautotrophically at 34 °C as described previously¹³. Transformant of wild type with *desA::Km^r* was cultivated in the same way but in the presence of 5 µg ml⁻¹ kanamycin in the culture medium. The disrupted gene, *desA::Km^r*, was constructed by interrupting the *desA* in the cloned Bluescript/1.5 kbp at the *Hind*III site by the aminoglycoside 3'-phosphotransferase gene (the kanamycin-resistance (Km^r) cartridge) originating from the bacterial transposon Tn5 (ref. 16). Fatty acids of the total membrane lipids were analysed according to Sato and Murata¹⁷. The values are the means obtained in three independent experiments, and the deviation of values was within ±1.0%.

* To whom correspondence should be addressed.

a

1 AATTCAGAGCAATTCGGTCTCTGGTCAATGGCAACGTGTATATAAAGAAAAGTTTGT
 61 TACCTGAGTATTAATTCCTAGGACAGGCAACCTTGGCCGCTTTATAGCCCATGAATCCA
 121 TAAACAAATCTGTCGACCTTCCATTGGAGATAAACCTTTATAA

167 ATGACTGCCACGATTCCCTCGTTGACACCAACGTAACGCCAGCAATCCGATCGCCCG
 1 MetThrAlaThrIleProProLeuThrProThrValThrProSerAsnProAspArgPro

227 ATTGCGGATCTCAAACTACAAGCATCATTAACACCTGCCAAGGAATGCTTCGAGAAA
 21 IleAlaAspLeuLysLeuGlnAspIleIleLysThrLeuProLysGluCysPheGluLys

287 AAAGCGAGCAAGCCTGGGCTCTGTTTGTATTACCTAGGGCGATCGCCGTGGGCTAT
 41 LysAlaSerLysAlaTrpAlaSerValLeuIleThrLeuGlyAlaIleAlaValGlyTyr

347 TTGGGCATTATTTATCTGCTCTGCTTGCCTTACCTGGATCTGGACAGGACA
 61 LeuGlyIleIleTyrLeuProTrpTyrCysLeuProIleThrTrpIleTrpThrGlyThr

407 GCCTTAACGGGGCTCTGTTGTGCGCCATGACTGTGGCCATCGCTCTTGTGTAATAA
 81 AlaLeuThrGlyAlaPheValValGlyHisAspCysGlyHisArgSerPheAlaLysLys

467 CGCTGGGCAATGATTAGTGGGACATATCGCTTTGCTCCCTCATCTACCCCTTCCAT
 101 ArgTrpValAsnAspLeuValGlyHisIleAlaPheAlaProLeuIleTyrProPheHis

527 AGCTGGGCTACTCCACGACCATCACCTCCACACCAACAAATGAGGTTGATAAC
 121 SerTrpArgLeuLeuHisAspHisHisHisLeuHisThrAsnLysIleGluValAspAsn

587 GCCTGGGATCCCTGGAGTGTGAAGCTTCCAAGCCAGCCGGGATCGTCGGGCTTTT
 141 AlaTrpAspProTrpSerValGluAlaPheGlnAlaSerProAlaIleValArgLeuPhe

647 TATCGGGCCATCCGGGCTCTGTTGTGAGCTGCTTCCATTTCCTAGGAGCTTAATG
 161 TyrArgAlaIleArgGlyProPheTrpTrpThrGlySerIlePheHisTrpSerLeuMet

707 CACTTCAAACTTCCAACTTTGCCAAAGGACCGCAATAAGTCAAATTATCCATTGCC
 181 HisPheLysLeuSerAsnPheAlaGlnArgAspArgAsnLysValLysLeuSerIleAla

767 GTTGCTCTCTGTTTGGCGGATCGCTTTCCTGCCCTAATTATCACCAGGGGTGTGG
 201 ValValPheLeuPheAlaAlaIleAlaPheProAlaLeuIleIleThrThrGlyValTrp

827 GGTTCGTCAAATTTTGGCTAATGCCCTGTTTGGTGTATCACTTTTGGATGAGCACTTT
 221 GlyPheValLysPheTrpLeuMetProTrpLeuValTyrHisPheTrpMetSerThrPhe

887 ACCATTGTGACCCACCATTCCTCGAAATTCGTTCCGTCCTCCGCGCGATGGAGTGCC
 241 ThrIleValHisHisThrIleProGluIleArgPheArgProAlaAlaAspTrpSerAla

947 GCCGAAGCCAGTTAAATGGTACTGTTCACTGCGATTATCCCGTTGGGTGGAAGTGCTC
 261 AlaGluAlaGlnLeuAsnGlyThrValHisCysAspTyrProArgTrpValGluValLeu

1007 TGCCATGACATCAACGTCCATATTCACCACACCTCTCCGTTGCCATCCCTTCCTATAAC
 281 CysHisAspIleAsnValHisIleProHisHisLeuSerValAlaIleProSerTyrAsn

1067 CTACGACTAGCCACCGAAGTTTAAAGAAAACCTGGGACCTTTTCTTTACGAGCGCAC
 301 LeuArgLeuAlaHisGlySerLeuLysGluAsnTrpGlyProPheLeuTyrGluArgThr

1127 TTTAACTGGCAATTAATGCAACAAATAGTGGCAATGTCATTATATGACCCGAACAT
 321 PheAsnTrpGlnLeuMetGlnGlnIleSerGlyGlnCysHisLeuTyrAspProGluHis

1187 GCCTACCGCACCTTCGGCTCCCTGAAAAAGTTTAATCTGGGACAACTAGTAATTTTGG
 341 GlyTyrArgThrPheGlySerLeuLysLysVal***

1247 ACCCATGATTGGTCAGTAATTAACCTTTGACTGATCCCCAGGGAGAGAAATACCATCAC
 1307 AAAATTAACTATCTTGAATGCGGCATCGAGCTGATCTCTTTTCTTTCTTGGTGAG
 1367 GAAAAAACTTTCTAAGTGGGACAGTGGGACGGTTCAGACTAGAAAGATCCACTCGGCC
 1427 AAAATGAATGCTAAACGCAACCTGCATATTCTCAAGGTTTCTACTAGCCAAGGTACCCC
 1487 TTC

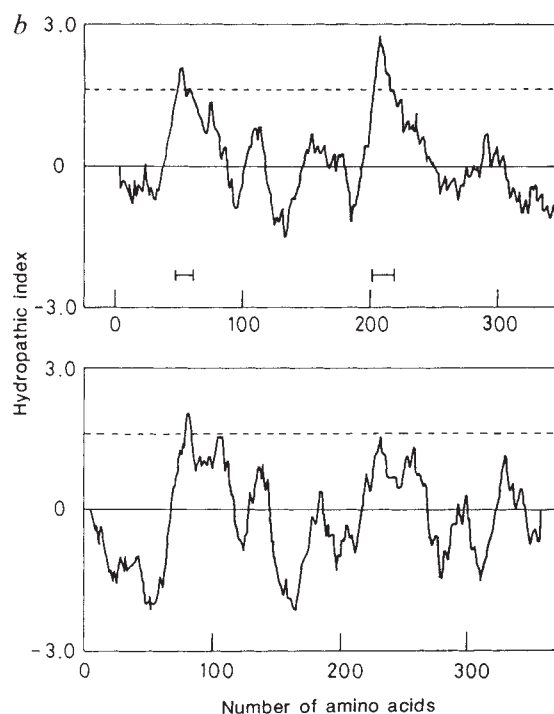


FIG. 1 *a*, Nucleotide sequence and deduced amino-acid sequence of *desA*, a gene for fatty-acid desaturation at the Δ^{12} position of fatty acids in *Synechocystis* PCC6803. The deduced amino-acid sequence is numbered with 1 for the first methionine. *b*, Hydropathy profiles of the deduced amino-acid sequences of the *desA* product and its putative membrane-spanning regions indicated by solid bars (top graph), and the stearyl-CoA desaturase from rat liver⁹ (bottom graph).

METHODS. Nucleotide sequence was determined by the dideoxy chain-termination method using double-stranded DNA templates¹⁸. The unidirectional deletion of the plasmid was performed according to the instructions of the manufacturer of the Bluescript DNA sequencing system (Stratagene Cloning Systems). Hydropathy index was calculated according to the algorithm of Kyte and Doolittle¹⁹ for a window size of 19 amino-acid residues.

To clone a gene required for the desaturation at the Δ^{12} position, a genomic library of *Synechocystis* PCC6803 was constructed in plasmid pTZ19R. The genomic library was screened for clones capable of complementing Fad12 in the growth at low temperature and the desaturation at Δ^{12} position according to the *in situ* transformation method developed by Dzelzkalns and Bogorad⁷. A plasmid clone with an 8-kilobase pair (kbp) insert, termed pTZ19R/8 kbp, was isolated (Table 1). The homologous recombinations as described by Williams⁸ between pTZ19R/8 kbp and the mutated gene of the chromosome of Fad12 may have taken place. The plasmid, pTZ19R/8 kbp, was digested with *Ava*I to obtain a 1.5-kbp fragment, which could also complement Fad12. The 1.5-kbp fragment was subcloned into a Bluescript plasmid (termed Bluescript/1.5 kbp), and its nucleotide sequence was determined. In only one of the six possible reading frames was there an open-reading frame. This was of 1,053 bp and corresponded to 351 amino-acid residues (Fig. 1*a*). The 1.5-kbp fragment also contained a 5' upstream region of 166 bp and a 3' downstream region of 270 bp. This gene (termed *desA*) encodes either a plant-type desaturase, which can introduce the second *cis* double bond at the Δ^{12} position of fatty acid bound to membrane glycerolipids, or a cofactor of this desaturase (see below). The hydropathy profile of the deduced amino-acid sequence of the *desA* product (Fig.

1*b*) is similar to that of the stearyl-CoA desaturase from rat liver⁹. The *desA* gene product has two clusters of hydrophobic regions which are putative membrane-spanning domains (Fig. 1*b*). But, the sequence similarity between the *desA* product and stearyl-CoA desaturase from rat liver is <30% at the nucleotide level and <10% at the amino-acid level.

We transformed the *Synechocystis* mutant, Fad12, with *desA* included in Bluescript/1.5 kbp and pTZ19R/8 kbp to examine whether the *desA* product is responsible for the fatty-acid desaturation. Table 1 shows that the wild type and the transformants contained high levels of 18:1 (9), 18:2 (9, 12) and 18:3 (6, 9, 12) fatty acids (fatty acids are represented by numbers of carbon atoms and double bonds, before and after a colon, respectively, and the positions of double bonds, counted from the carboxy terminus (Δ), are indicated by numbers in parentheses). In Fad12, 18:1 (9) and 18:2 (6, 9) significantly increased, whereas 18:2 (9, 12) and 18:3 (6, 9, 12) decreased to trace amounts. It is noteworthy that Fad12 lacked the fatty acids having the double bond at the Δ^{12} position, and that this double bond was recovered by transformation with *desA*. Similar changes in the desaturation of fatty acids at the Δ^{12} position were observed in all lipid classes, monogalactosyl diacylglycerol, digalactosyl diacylglycerol, sulfoquinovosyl diacylglycerol and phosphatidylglycerol (data not shown). To examine whether

the *desA* gene product is necessary for fatty-acid desaturation, we transformed the wild-type *Synechocystis* PCC6803 according to Williams⁸ with a disrupted *desA*, which was produced by insertion of a kanamycin-resistance cartridge (Km^r). The transformant of wild type with *desA::Km^r* had the same fatty-acid composition as that of Fad12 (Table 1).

Another transformable cyanobacterium, *Anacystis nidulans* R2-SPc, was transformed with *desA* according to Kuhlemeier and van Arkel¹⁰. It is noteworthy that *A. nidulans* is a member of the group of cyanobacteria, which are completely defective in desaturation at the Δ^{12} position^{11,12}. Bluescript/1.5 kbp was digested with *SacI* to obtain a fragment containing the total sequence of the 1.5-kbp insert. This fragment was subcloned into pUC303 (ref. 10), a shuttle vector between *A. nidulans* and *Escherichia coli*, at the *SacI* site of the streptomycin-resistance gene but in the opposite direction (termed pUC303/*desA*). The wild-type and the transformant with pUC303 alone (control) contained 16:0, 16:1 (9), 18:0 and 18:1 (9) as the principal fatty acids, indicating that this cyanobacterium can introduce only one double bond into the C_{16} and C_{18} fatty acids (Table 2). In the transformant with pUC303/*desA*, fatty acids having two double bonds, 16:2 (9, 12) and 18:2 (9, 12), emerged to significant levels at the expense of 16:1 (9) and 18:1 (9). The transformation of *A. nidulans* R2-SPc with the disrupted *desA* by the Km^r cartridge was also carried out as above. The transformant with pUC303/*desA::Km^r* had a fatty-acid composition similar to that of the wild type and the transformant with pUC303. The lipid class composition and the lipid-to-protein ratio were not affected by transformation with pUC303 and pUC303/*desA*. These observations demonstrate that the transformant with *desA* has acquired the desaturase activity in

TABLE 2 Composition of major fatty acids of total membrane lipids in various strains of *Anacystis nidulans* R2-SPc

Strain	Fatty acid (mol %)					
	16:0	16:1 (9)	16:2 (9, 12)	18:0	18:1 (9)	18:2 (9, 12)
Wild type	51	36	0	3	6	0
Transformant with pUC303	51	37	0	3	5	0
Transformant with pUC303/ <i>desA</i>	47	29	5	5	2	6
Transformant with pUC303/ <i>desA::Km^r</i>	50	33	0	5	9	0

Wild type was grown photoautotrophically at 34 °C as described previously¹³. Transformants with pUC303 and pUC303/*desA* were grown in the same way as above but in the presence of 7.5 $\mu\text{g ml}^{-1}$ chloramphenicol. Transformant with pUC303/*desA::Km^r* was grown as wild type but in the presence of 7.5 $\mu\text{g ml}^{-1}$ chloramphenicol and 5 $\mu\text{g ml}^{-1}$ kanamycin. The disrupted gene, pUC303/*desA::Km^r*, was constructed by interrupting *desA* in the cloned pUC303/*desA* at the *Bam*HI site by the Km^r cartridge. Fatty acids were analysed according to Sato and Murata¹⁷. The values are the means obtained in three independent experiments, and the deviation of values was within $\pm 1.0\%$. Ten independently obtained transformants with *desA* gave the same result as above.

introducing the second double bond at the Δ^{12} position of fatty acids, and that the 166-bp upstream sequence contains the promoter region of this gene.

A. nidulans is sensitive to chilling temperature^{3,4,13}. At growth temperature, both plasma and thylakoid membranes are in the

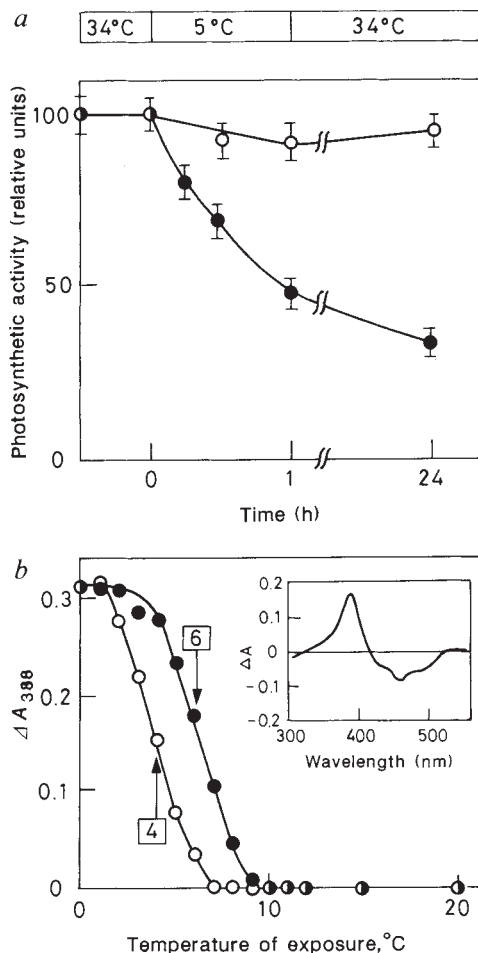


FIG. 2 Effects of low temperature on the photosynthetic activity and phase transition of membrane lipids in the transformants with and without *desA* of *Anacystis nidulans* R2-SPc. ●, Transformant with pUC303; ○, transformant with pUC303/*desA*. a, Effect of exposure to 5 °C on the photosynthetic activity. The activity before exposure to 5 °C corresponded to about 190 $\mu\text{mol O}_2$ per mg chlorophyll per h for both transformants. Each point represents the mean $\pm$ s.d. obtained in three independent experiments. b, Effect of exposure to low temperature for 20 min on the absorbance change at 388 nm. Inset: changes in absorption spectrum caused by exposure to 6 °C for 20 min in the transformant with pUC303 at a concentration corresponding to 15 $\mu\text{g chlorophyll ml}^{-1}$. Two independently obtained transformants gave essentially the same result.

METHODS. Cells grown at 34 °C were exposed to low temperature for 20 min in the dark. At an appropriate time the cells were rewarmed to 34 °C, and the photosynthetic activity (light-included oxygen evolution) and the absorption spectrum were measured according to Ono and Murata¹³.

liquid-crystalline state. With decrease in growth temperature, the thylakoid membrane first goes into the phase-separated state only with reversible deterioration of photosynthesis. On further decrease in temperature, the plasma membrane enters the phase-separated state, in which leakage of the cytosolic solutes of low relative molecular mass into the medium irreversibly damages physiological activities^{3,4}.

When the wild type and transformant with pUC303 of *A. nidulans* R2-SPc grown at 34 °C were exposed to 5 °C for 60 min, more than 50% of photosynthetic activity was lost, and further decline of the activity continued after transferring them to 34 °C (Fig. 2a). By contrast, the transformant with pUC303/*desA* did not lose photosynthetic activity during the exposure to 5 °C for 60 min (Fig. 2a). On exposure to 2 °C for 60 min, the photosynthetic activity was decreased to 40% in the transformant with pUC303 and to 75% in the transformant with pUC303/*desA*. These observations demonstrate that chilling tolerance of *A. nidulans* R2-SPc was enhanced by transformation with *desA*.

The phase transition from the liquid-crystalline to the phase-separated state of the plasma membrane in intact cells of *A. nidulans* can be studied by changes in the absorption spectrum of carotenoids^{3,13-15}. The phase transition of the plasma membranes of the transformants containing pUC303 or pUC303/*desA*, both grown at 34 °C, appeared in temperature ranges 8–4 °C with a midpoint at 6 °C, and 6–2 °C with a midpoint at 4 °C, respectively (Fig. 2b). The lowering in the phase transition temperature of the plasma membrane by *desA* can be regarded as resulting from the introduced desaturase activity,

and this provides a molecular basis for the enhancement of chilling tolerance of this cyanobacterium.

The present study demonstrates that the chilling tolerance of cyanobacteria can be enhanced by genetic manipulation of fatty-acid desaturation. Because a similar mechanism could operate in the chilling injury of higher plants, it might be possible to improve their chilling tolerance by similarly manipulating fatty-acid desaturation. □

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Partition of tRNA synthetases into two classes based on mutually exclusive sets of sequence motifs

Gilbert Eriani, Marc Delarue*, Olivier Poch, Jean Gangloff & Dino Moras*

Laboratoires de Biochimie et de * Cristallographie Biologique, IBMC du CNRS, 15 rue Rene Descartes, 67084 Strasbourg, France

THE aminoacyl-transfer RNA synthetases (aaRS) catalyse the attachment of an amino acid to its cognate transfer RNA molecule in a highly specific two-step reaction. These proteins differ widely in size and oligomeric state, and have limited sequence homology. Out of the 18 known aaRS, only 9 (ref. 1), referred to as class I synthetases (GlnRS, TyrRS, MetRS, GluRS, ArgRS, ValRS, IleRS, LeuRS, TrpRS), display two short common consensus sequences ('HIGH' and 'KMSKS') which indicate, as observed in three crystal structures²⁻⁴, the presence of a structural domain (the Rossman fold) that binds ATP. We report here the sequence of *Escherichia coli* ProRS, a dimer of relative molecular mass 127,402, which is homologous to both ThrRS and SerRS. These three latter aaRS share three new sequence motifs with AspRS, AsnRS, LysRS, HisRS and the β subunit of PheRS. These three motifs (motifs 1, 2 and 3), in a search through the entire data bank, proved to be specific for this set of aaRS (referred to as class II). Class II may also contain AlaRS and GlyRS, because these sequences have a typical motif 3. Surprisingly, this partition of aaRS in two classes is found to be strongly correlated on the functional level with the acylation occurring either on the 2' OH (class I) or 3' OH (class II) of the ribose of the last nucleotide of tRNA.

To clone the ProRS gene, a pool of partially digested *E. coli* DNA fragments was used to transform and complement the strain UQ27 (*proS27*, *argG*, *lac*, *thi*), a temperature-sensitive mutant defective in ProRS activity⁵. From complementing trans-

formants we isolated several types of plasmids, whose insert sizes were reduced by limited *Sau3A* digestion, ligated into plasmid pUC18 vector, and selected in strain UQ27 at 43 °C. The resultant bacterial cells overproduced by 100 times the ProRS activity of wild-type cells. A 2.8-kilobase (kb) DNA fragment was subcloned into M13mp18, partially digested with exonuclease⁶ and sequenced using modified T7 DNA polymerase⁷. The 2,795 base pairs (bp) that were sequenced contain an open reading frame encoding a protein of 572 residues whose relative molecular mass (M_r 63,701) is in good agreement with that estimated by SDS-PAGE (data not shown). The N-terminal protein sequence deduced from the primary structure was independently confirmed by sequencing the first 12 N-terminal residues of the purified ProRS. In addition, the *proS* messenger RNA 5' termini were determined as described in Fig. 1. Analysis of the DNA region downstream of the TGA stop signal revealed a G+C-rich sequence of hyphenated dyad symmetry, centred on position +1,739 and followed by a run of seven T residues. This structural feature corresponds to a rho-independent termination signal, as indicated by the University of Wisconsin Genetic Computer Group (UWGCG) TERMINATOR program using the algorithm of Brendel and Trifonov⁸, which predicted a stop site for RNA polymerase at position +1,755 shown in Fig. 1.

The comparison of the ProRS sequence with other aaRS sequences showed extensive homologies with ThrRS (19.2% of strict identity and 44.8% of conservative substitutions as defined in the legend of Fig. 2, not considering the 110 amino-acid insert of ProRS) and with SerRS (13.7% of strict identity and 37.2% of conservative substitutions). An alignment of these three proteins is presented in Fig. 2. ProRS and ThrRS have both longer C-terminal domains than SerRS, whereas ThrRS has a long extension at its N terminus, as compared with ProRS and SerRS. Is this extension implicated in the autoregulation of the ThrRS translation, a unique feature of this aaRS? As already shown⁹, this mechanism proceeds through an interaction between the ThrRS and a tRNA^{Thr} anticodon-like structure located upstream of the AUG initiation codon of *thrS*. More recently, however, several results¹⁰ pointed to the existence, at the level of *thrS* operon, of a further domain interacting with ThrRS and whose

primary structure has no homologies with tRNA^{Thr}; the N-terminal extension of ThrRS, which can be reasonably assumed not to be implicated in catalysis and, by consequence, in tRNA binding, is a good candidate for containing this second site of interaction with the *thrS* operator.

Systematic pairwise comparisons were then made between the sequences of ProRS, SerRS and ThrRS on one hand and all the other aaRS sequences of *E. coli* on the other. Three regions of high homology appeared in the following pairs: AsnRS with SerRS, ProRS with AspRS, and ProRS with HisRS. Two of these regions have already been noted to be present in AspRS, AsnRS, LysRS and HisRS and one in PheRS as well¹¹⁻¹⁵. Here we show that there is another region of high sequence homology and that the three of them can also be found in SerRS, ThrRS and ProRS. All the known sequences for the eight different synthetases described above, but isolated from organisms other than *E. coli*, were analysed and the three regions of homology were detected and aligned. To test for consistency, a 'profile' (sequence probe constituted from a group of aligned sequences as defined by Gribskov *et al.*¹⁶) was constructed from the alignment of the three motifs of only AsnRS, AspRS, LysRS, HisRS and the β subunit of PheRS. This profile, in a search through the entire protein database, proved to be valid¹⁶ and

able to detect at an 'interesting level' (see Gribskov *et al.*¹⁶) the ThrRS. On the other hand, the nine members of class I aaRS (MetRS, TyrRS, GlnRS, GluRS, ArgRS, TrpRS, IleRS, LeuRS and ValRS) were never detected in our profile searches through the data bank, using the three motifs of class II, either alone or concatenated. An alignment of these three motifs is presented in Fig. 3.

Each motif contains a strongly conserved core with an invariant residue: g ϕ xx ϕ xxP ϕ ϕ for motif 1; (F/Y/H)Rx(E/D) followed 4 to 12 residues away by (R/H)xxxFxxx(D/E) for motif 2; and λ x ϕ g ϕ g ϕ eR ϕ ϕ ϕ ϕ for motif 3 (see legend of Fig. 2 for definition of symbols: x stands for any residue, single letter amino-acid code is used, with lower case for residues conserved in at least 11 out of the 17 possible sequences). The distance between the first and the second motifs is relatively constant (between 40 and 80 residues), whereas the distance between the second and the third motifs is more variable (between 70 and 300 residues), suggesting that an entire domain can be inserted in this intermotif region. In most cases, the third motif is located very close to the C-terminal end.

AlaRS (a tetramer) and GlyRS ($\alpha_2\beta_2$) can be also assigned to class II, because they have an almost canonical motif 3 (ref. 11); in addition, motif 3 in AlaRS is located in the smallest

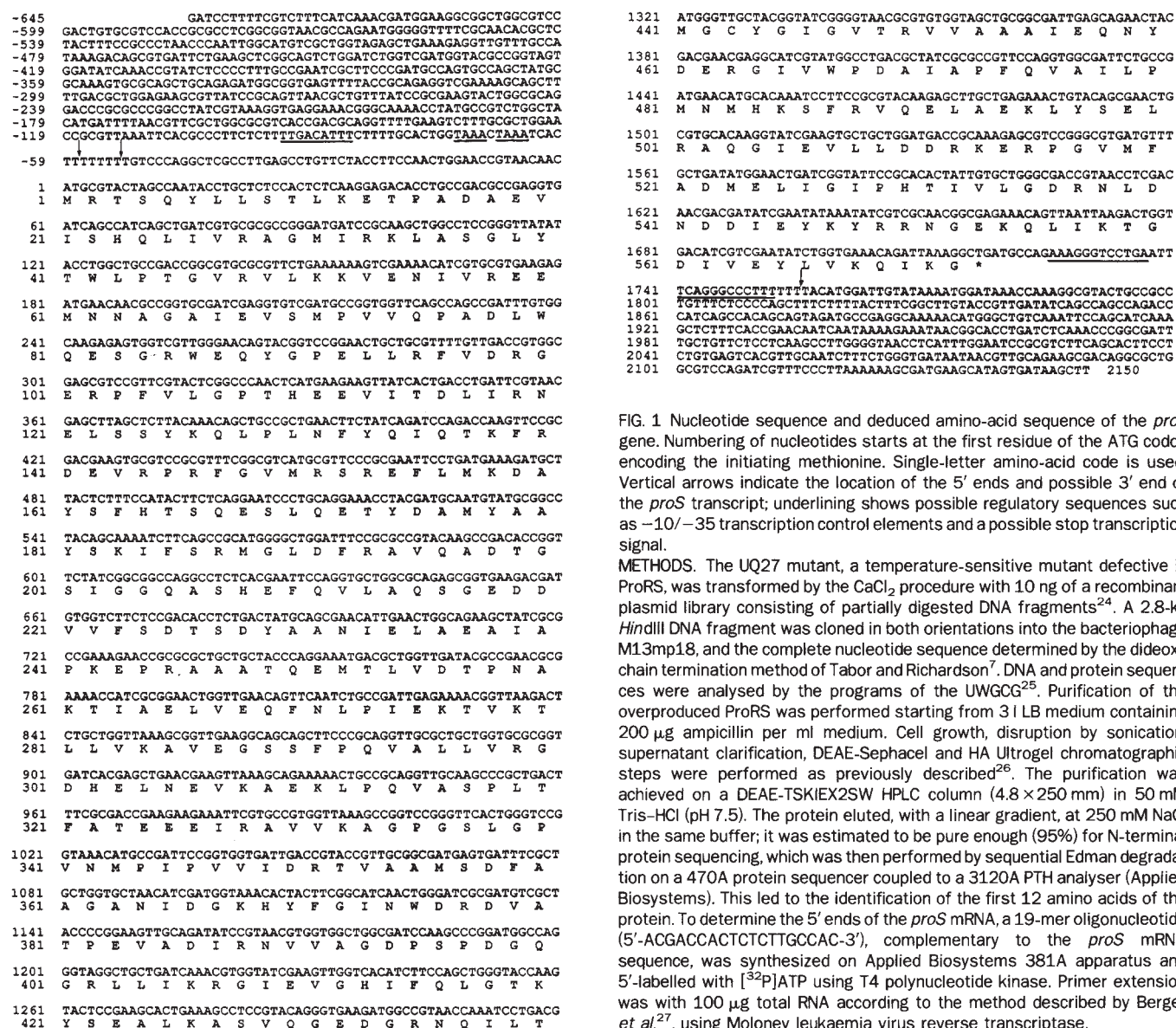


FIG. 1 Nucleotide sequence and deduced amino-acid sequence of the *proS* gene. Numbering of nucleotides starts at the first residue of the ATG codon encoding the initiating methionine. Single-letter amino-acid code is used. Vertical arrows indicate the location of the 5' ends and possible 3' end of the *proS* transcript; underlining shows possible regulatory sequences such as -10/-35 transcription control elements and a possible stop transcription signal.

METHODS. The UQ27 mutant, a temperature-sensitive mutant defective in ProRS, was transformed by the CaCl₂ procedure with 10 ng of a recombinant plasmid library consisting of partially digested DNA fragments²⁴. A 2.8-kb *Hind*III DNA fragment was cloned in both orientations into the bacteriophage M13mp18, and the complete nucleotide sequence determined by the dideoxy chain termination method of Tabor and Richardson⁷. DNA and protein sequences were analysed by the programs of the UWGCG²⁵. Purification of the overproduced ProRS was performed starting from 3 l LB medium containing 200 μ g ampicillin per ml medium. Cell growth, disruption by sonication, supernatant clarification, DEAE-Sephacel and HA Ultragel chromatographic steps were performed as previously described²⁶. The purification was achieved on a DEAE-TSK/EX25W HPLC column (4.8 $\times$ 250 mm) in 50 mM Tris-HCl (pH 7.5). The protein eluted, with a linear gradient, at 250 mM NaCl in the same buffer; it was estimated to be pure enough (95%) for N-terminal protein sequencing, which was then performed by sequential Edman degradation on a 470A protein sequencer coupled to a 3120A PTH analyser (Applied Biosystems). This led to the identification of the first 12 amino acids of the protein. To determine the 5' ends of the *proS* mRNA, a 19-mer oligonucleotide (5'-ACGACCACTCTCTGCCAC-3'), complementary to the *proS* mRNA sequence, was synthesized on Applied Biosystems 381A apparatus and 5'-labelled with [³²P]ATP using T4 polynucleotide kinase. Primer extension was with 100 μ g total RNA according to the method described by Berger *et al.*²⁷, using Moloney leukaemia virus reverse transcriptase.

FIG. 2 Total alignment between *E. coli* ProRS (P), SerRS (S) and ThrRS (T), obtained by the Needleman and Wunsch algorithm, as implemented in P. Argos' program²⁸ for pairwise comparisons; the alignment of the three proteins was then manually adjusted. Conserved residues in the three sequences are indicated by: single-letter code for the invariant residue; λ for small amino acids P, G, S, T; - for negatively charged amino acids D, E, N, Q; + for positively charged amino acids H, R, K; and φ for hydrophobic residues F, Y, W, I, L, V, M, A. The motifs 1, 2 and 3 as shown in Fig. 3 are bold and underlined.

T 78 LLGHAIKQLWPHTKMAIGPVIDNGFFYYDVLDRTLTQEDVEALEKRMHLEAKNYDVIK
S 1 MLDPNL..LRNEPDAVÆKLARRGFKLDVD.....KLGALÆERKVLQVKTENLQAE

T 138 KVSWEARETFANRGES....YKVSILDENIAHDDKPGLYFHÆEYVDMC.RGPHVPMNR
S 51 RNSRSKSIGQAKARGEDIEPLRLÆVNLKGEELDAKÆLDALQAEIRDIALTIFNPDADE

D φλ

P 1 MRTSQYLLSTLKETPADAEVISHQL
T 192 FCHRFKLMKTAGAYWRGDSNNKMLQRIYGTAWADKKALNAYLQRLÆAAKRDHRKIGKQL
S 111 VPVGKDENDNÆVEVSRWGTP.....REFDFVRDHTLGMH

φ φ λG φ G - φφ - φ E Pφφ φ

P 26 .IVRAGMIRKLASGLYTWLP TGVR...VLKKVENIVREEMNAGAIÆVSMFVQPADLWQ
T 252 ...DLYMHQÆEAPGMVÆWHNDGWT...IFRELEVFRSKLEKYQQÆVKGFFMDRVLWE
S 147 SGLDFAAAVKL.TGSRFVVMKGQIARMHRALSQFMLDLRTEQHGYSENYVPIVLNQDTLY

λG φ λG φ φ P - φφ φ - LPφ φ λ

P 82 ESGRWEQYGPPELLRFV....DRGERPFVLGTTHÆEVI TDLIRNELSSYKQLLNFIYQIQT
T 306 KTGHWNDYKDMÆFTTS....SENREYCIKPMNCPHGVQIFNQGLKSRYDLLRMAÆFGS
S 206 GTGQLPFGAGDLFHTFRLÆEÆADTSNYALIPTÆEVLPTNLVRÆIIEDDLLRKMTAHTP

R E λ GφφR + F - φ φ -- φ φ φ φ φGφ

P 138 KFRDEVRPRF...GVMRSRFLMKDAYSFHTSQESLQETDYDAMAAYSKIFSRMGDLFR
T 361 CHRNÆPGSLH...GLMRVRFGTQDDAHIFCTEQIRDEVNGCIRLVY.DMYSTFGÆKI
S 266 CFRSEAGSYGRDTRAGLIRMHQFDKVMÆVQIVRPEDSMA.ALEEMTGHAÆKVLQLLGLPYR

φ φ T

P 193 AVQA...DTGSIGGSASHEFQVCAQSGEDDVVFSDTSDYAANIÆLÆLIAPEKPRÆATQÆ
T 417 VVKLVKLSTRÆKRIGSDÆMWDRAEDLVALEENNIPFEYQLGEGAFYGPK.....
S 325 KIIL...CTGDMGFGA.....YRÆISS

Y T

P 362 - 110 aa - GANIDGKHYYFINWRDVTPEVADIRNVVAGDPSPDGQGRLLYKRGIE
T 466IETFLYDCLNRAQCGT.....
S 338CKTYDLEWIPAQNT.....YRÆISS

φ-φ S λ- φ Rφφ φ E- φP

P 411 VGHIFQLGTKYSEALKASVQGEDG..RNQLTMCGYIGIVTRVAAAEQNYDERG.IVNE
T 483VQLDFSLPSRLSASYGEDNERKVPVMIHRAILGSMERFIGILTEEFAG....FFP
S 359 CSNVWDFQARRMQARCRS.KSDKK..TRLVHTLNGSLAVGRTLIVAVMENYQADGRIEVE

φφ Pφ φ φ

P 469 DAIAFPQVAILPMMHKSFRVQELÆAKLYSELRAQGIEVLDDRKRPFVGMFADMELIG
T 535 TWLÆPQVVIMINITSQS...EYVNELTQKLSNAGIRVKADLRNÆKIGFKIREHTLRR
S 417 EVLRPYMNGLEYIG 430

P 528 IPHTIVLGDNRNLNDNDIEYKYRRNGEKQLIKTGDIÆYVLVKQIKG 572
T 590 VPYMLVCGDKEVESGKVAVRTRRGKDLRDMVDNÆVEIEKLOQÆIRSRSLKQLEE 642

[illegible]

FIG. 3 Actual alignment in the motifs region for class II synthetases. The package UWGCG²⁵ (version 6.1, August 1989) and the data banks included therein were used to extract all the known sequences of aaRS. For a given position, when more than 11 (out of 17 possible) of the residues were identical (underlined) or belonged to the same group, a symbol, as defined in Fig. 2, was written on the top line. The position of the first amino acid shown is indicated as well as the distance between the motifs. The sequences are (the UWGCG access code for each protein data file, where key references can be found, is given in parentheses): LysRS from *E. coli*¹⁴ (ECO) and *S. cerevisiae*²⁹ (YST); AspRS from *E. coli*¹⁹, rat (RAT) (Un: j04487) and *S. cerevisiae* (Pl: yscats); HisRS from *E. coli* (Ba:ecohiss), hamster (HAM) (Ro:hamhrrs1) and *S. cerevisiae* (Pl: yschts1); PheRS from *E. coli* (beta subunit, Ba:ecothrinf), from mitochondria (MIT) (Pl: yscmsf) and cytoplasm (beta subunit) of *S. cerevisiae*; AsnRS from *E. coli*¹³, ThrRS from *E. coli* (Ba:ecothrinf) and *S. cerevisiae* (Pl: yschts1); ProRS from *E. coli* (this work);

SerRS from *E. coli* (Ba:ecoserS) and *S. cerevisiae* (Pl:yscserS); AlaRS from *E. coli* (Ba:ecoalas; and the β subunit of GlyRS from *E. coli* (Ba:ecoglyS). The motifs shown were used to screen the entire protein database, using a program described by Gribskov *et al.*¹⁶. A profile constructed from the three motifs aligned in all the eight different synthetases was first validated because it detected, at the required level (above 2.2 σ , as advocated by Gribskov *et al.*¹⁶), all the sequences that were used to build it; no additional protein was detected. But a profile derived only from motif 1 or from motif 2 detected a fragment of a protein (code qqcfcfr), as already noted by Gampel and Tzagoloff¹²; this protein is almost certainly an aaRS. Motif 3 alone detected with a convincing score AlaRS and the β subunit of GlyRS. Motif 3 alone and motif 1 alone also detected a potentially protective antigen from lymphatic filariasis (code A28209 (ref. 30)), which is homologous to AspRS (T. Nilsen, personal communication). The homology with AsnRS from *E. coli* is even higher (35% strict identity).

TABLE 1 Partition of aaRS into class I and class II on the basis of presence or absence of three motifs*

Class II synthetases		Class I synthetases	
Motif 3 only	Motifs 1, 2, 3	HIGH + KMSKS	
Gly ($\alpha 2\beta 2$) 3' OH			
Ala ($\alpha 4$) 3' OH			
	Pro ($\alpha 2$) 3' OH		
	Ser ($\alpha 2$) 3' OH no RF		
	Thr ($\alpha 2$) 3' OH		
	Asp ($\alpha 2$) ??		
	Asn ($\alpha 2$) 3' OH		
		Glu (α) 2' OH	
		Gln (α) ?	RF
	His ($\alpha 2$) 3' OH		
	Lys ($\alpha 2$) 3' OH		
		Arg (α) 2' OH	
		Val (α) 2' OH	
		Ile (α) 2' OH	
		Leu (α) 2' OH	
		Met ($\alpha 2$) 2' OH	RF
		Tyr ($\alpha 2$) ??	RF
		Trp ($\alpha 2$) ?	
	Phe ($\alpha 2\beta 2$) 2' OH		

* Motifs are shown in Fig. 3.

The oligomeric state of the active form of the enzyme is shown in parentheses. The primary site of aminoacylation is also indicated²⁰. This site is evolutionarily conserved (*E. coli* and *Saccharomyces cerevisiae*) except for TrpRS and GlnRS (question mark). For CysRS (not shown here, as its sequence is not known), AspRS and TyrRS, both sites seem to be possible (??). For those aaRS whose three-dimensional structure is known, the presence or absence of the Rossman fold (RF) is indicated. The table has been arranged to take into consideration both the volume (increasing towards the bottom) and the chemical nature of the residues.

engineered fragment (1-385; ref. 17) able to activate alanine. None of the class II aaRS contain the HIGH and KMSKS signature sequences characteristic of class I and indicative of the presence of the Rossman fold. The recent crystal-structure determination of SerRS (a member of class II) indicates that this protein does not contain the Rossman fold (S. Cusack and R. Lebermann, personal communication).

Secondary structure predictions indicate that the two strictly conserved arginine residues of motifs 2 and 3 are located in loops or turns. Motifs 1, 2 and 3 may then cooperate to build

a functional domain involved in the aminoacylation and form specific (super)secondary structure(s) which define functionally important loops bearing the invariant residues. The functional importance of our motifs is confirmed by site-directed mutagenesis studies on the aspartic system. In yeast, deletion of the C-terminal part of the protein, close to motif 3, resulted in a dramatic loss of enzyme activity, and insertion of a dipeptide at position 299 (15 residues upstream of motif 2) had the same effect¹⁸. Similarly, the catalytic activities of the *E. coli* enzyme are drastically affected by the double mutation (FA229, DN233) in motif 2 (ref. 19).

The primary attachment of the activated aminoacyl to the last residue of tRNA can occur either on the 2' OH or the 3' OH of the ribose²⁰. Strikingly, we observed that synthetases that aminoacylate on the 2' OH almost invariably (except for PheRS) belong to class I, whereas those that aminoacylate on the 3' OH belong to class II (Table 1). Therefore, class I and class II synthetases can be tentatively characterized at the structural level as the ones that do and do not contain the Rossman fold, respectively, and, at the functional level, as the ones whose primary site of aminoacylation on the tRNA is a 2' OH or 3' OH, respectively. These results suggest that the catalytic domains of these two classes have evolved from two different ancestors containing (1) the Rossman fold for class I (which was probably already available in other proteins at the time when proteins gradually took over the job done by the very first aaRSs, which were probably catalytic RNAs²¹) and (2) another type of catalytic domain for class II, characterized by the three sequence motifs shown in Fig. 3 (the topology of this domain may be present in other proteins). However, these results do not exclude the possibility that aaRS of two different classes nonetheless share other structural features ('building blocks') different from the ones directly involved in the catalytic reaction.

Table 1 reveals two general trends: (1) the acylation of small and neutral residues (Ala, Gly, Pro, Ser and Thr) is by class II aaRS, whereas acylation of hydrophobic residues (except for PheRS) involves class I aaRS; and (2) for two amino acids of similar chemical nature, the smallest is activated by a class II and the largest by a class I aaRS (for example, Lys versus Arg, Asp versus Glu). The reasons for the specificity of aminoacylation by class I or class II aaRS are probably more complex but seem to have been governed so as to allow for the best discrimination against other amino acids of similar chemical nature and/or volume. The existence of two different classes of aaRS, as well as residue volume considerations^{22,23}, might have been evolutionarily advantageous and important for the emergence of an efficient proof-reading mechanism for the translation of the genetic code. □

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Requirement for GTP hydrolysis in the formation of secretory vesicles

Sharon A. Tooze, Ursula Weiss & Wieland B. Huttner*

Cell Biology Programme, European Molecular Biology Laboratory,
PO Box 10.2209, D-6900 Heidelberg, FRG

* To whom correspondence should be addressed

THE specificity of vesicular transport in a cell is determined by the formation of vesicles with specific contents from a donor compartment and their selective fusion with the appropriate acceptor compartment. Several of the latter fusion steps have been investigated in detail using cell-free systems¹⁻⁹, and work with these systems as well as genetic evidence has revealed a role for GTP-binding proteins in membrane fusion processes⁷⁻¹⁵. We have reconstituted the formation of constitutive secretory vesicles and immature secretory granules from the *trans* Golgi network in a cell-free system¹⁶. We show here that the budding of both types of post-Golgi vesicles is inhibited by non-hydrolysable analogues of GTP, which suggests a more widespread role for GTP-binding proteins in membrane traffic than previously assumed.

Incubation of a post-nuclear supernatant from PC12 cells in the presence of ATP results in the formation of constitutive secretory vesicles, identified by the presence of a sulphate-labelled heparan sulphate proteoglycan, and immature secretory granules, identified by the presence of sulphate-labelled secretogranin II (ref. 16). To investigate whether GTP-binding proteins are involved in the budding of vesicles from donor compartments, as they are in the fusion of vesicles with acceptor compartments⁷⁻¹⁵, we examined the effect of GTP γ S, a non-hydrolysable analogue of GTP, on the cell-free formation of immature secretory granules and constitutive secretory vesicles. As shown in Table 1, addition of 1 μ M GTP γ S inhibited the formation of both immature secretory granules and constitutive secretory vesicles by ~60%.

To investigate whether the inhibition of post-Golgi secretory vesicle formation by GTP γ S could be alleviated by GTP, 1 mM GTP was added before 1 μ M GTP γ S. The addition of GTP, which by itself did not stimulate the formation of immature secretory granules and constitutive secretory vesicles (data not shown), largely prevented the inhibition by GTP γ S of post-Golgi secretory vesicle formation (Table 1). At a 10-fold higher ratio of GTP γ S (10–20 μ M) to GTP (1–2 mM), the inhibitory effect of the non-hydrolysable analogue was only slightly

decreased by the presence of GTP (data not shown). The latter observations on secretory vesicle budding are similar to those made in a cell-free system in which the fusion of vesicles derived from the endoplasmic reticulum with the *cis* Golgi has been studied; these investigators found that 1 mM GTP prevented the inhibition of vesicle fusion by 1 μ M, but not 10 μ M, GTP γ S¹⁴.

On a molar basis, GTP γ S is a more effective inhibitor of vesicle fusion events than another non-hydrolysable analogue of GTP, GMP-PNP (ref. 11). Similarly, the formation of post-Golgi secretory vesicles was inhibited by 100 μ M GMP-PNP (Table 2) but not by 20 μ M (data not shown). The inhibition by non-hydrolysable GTP analogues was specific with respect to the guanine moiety as the addition of 50 μ M ATP γ S had no inhibitory effect (Table 2).

For both immature secretory granules (Fig. 1a) and constitutive secretory vesicles (Fig. 1b), the inhibition of formation by GTP γ S (used at 10 μ M) was observed at the earliest time point (30 min) at which this inhibition could be reliably quantitated. When the incubation was continued for 120 min, the extent of inhibition of formation of both types of post-Golgi secretory vesicles did not increase with time.

The extent of inhibition of both immature secretory granule and constitutive secretory vesicle formation did not increase significantly when higher concentrations of GTP γ S (20, 40 and 80 μ M) were used (data not shown); the mean inhibition was 64% and 73%, respectively, which was in the same range as that observed using 1 μ M GTP γ S. Under the various conditions used in this study (1–80 μ M GTP γ S, 30–120 min incubation), the average inhibition of post-Golgi secretory vesicle formation in nine separate experiments was 50% (ranging from 30–69%) for immature secretory granules and 65% (ranging from 40–94%) for constitutive secretory vesicles. The partial inhibition by GTP γ S of both immature secretory granule and constitutive secretory vesicle formation on *in vitro* incubation of the post-nuclear supernatant is similar to that observed for certain cell-free vesicle fusion processes^{7,15}; it may be explained by assuming that in some of the molecule(s) through which GTP γ S exerts its inhibitory effects, the GTP-binding site is already occupied by endogenous GTP, which does not exchange with the added GTP γ S, at the start of incubation.

Several lines of evidence indicate that the inhibitory effects of GTP γ S reported here occurred directly on budding reactions,

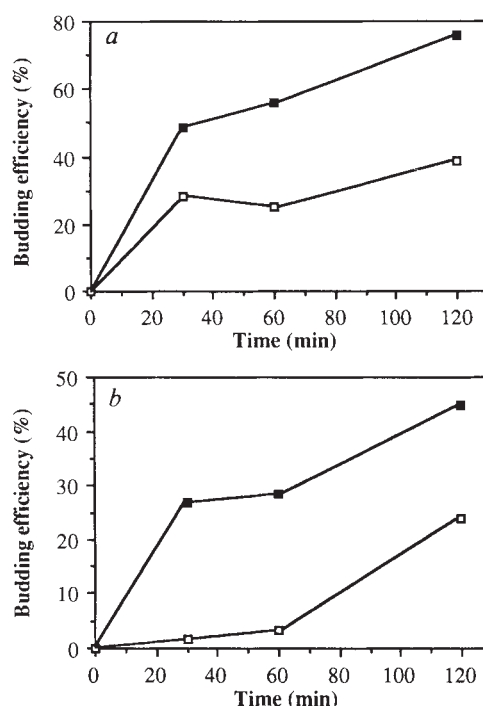


FIG. 1 Time course of GTP γ S inhibition of the formation of immature secretory granules (a) and constitutive secretory vesicles (b). Cell-free formation of both immature secretory granules and constitutive secretory vesicles was performed as previously described¹⁶, except that samples were incubated in the absence (■) or presence (□) of 10 μ M GTP γ S as described in Table 1. After incubation at 37 °C for 30, 60, or 120 min, the samples were analysed and the budding efficiencies calculated as described in Table 1.

TABLE 1 GTP γ S inhibits cell-free formation of post-Golgi secretory vesicles

	ISG		CSV	
	Budding efficiency	% Of control	Budding efficiency	% Of control
Control	36.3	100	29.6	100
GTP γ S (1 μ M)	14.2	39	11.3	38
GTP (1 mM) + GTP γ S (1 μ M)	29.5	81	21.7	73

Cell-free formation of both immature secretory granules (ISG) and constitutive secretory vesicles (CSV) was performed as previously described¹⁶, except that samples were incubated in the absence or presence of 1 μ M GTP γ S and 1 mM GTP as indicated. A post-nuclear supernatant was prepared from sulphate-labelled PC12 cells. GTP γ S (10 mM stock, Boehringer) and GTP (100 mM stock, Sigma) were added to the post-nuclear supernatant at 4 °C; GTP was added before the non-hydrolysable analogue. The post-nuclear supernatant was then incubated at 37 °C for 60 min for cell-free formation of secretory vesicles¹⁶. Immature secretory granules and constitutive secretory vesicles were separated from the *trans* Golgi network by velocity-controlled sucrose gradient centrifugation¹⁶, and the gradient fractions analysed by SDS-PAGE and fluorography. The budding efficiency of ISG was determined by quantitation of sulphate-labelled secretogranin II using liquid scintillation counting and is expressed as per cent of the starting material in the *trans* Golgi network, as previously described (see Fig. 7 of ref. 16). The budding efficiency of CSV was determined as for immature secretory granules by quantitation of the sulphate-labelled heparan sulphate proteoglycan, except that densitometric scanning was used for quantitation and that the sum of the value for each velocity gradient were normalized to that of the untreated control left at 4 °C. Budding efficiencies are also expressed as per cent of control.

as opposed to indirectly on some fusion reaction required for budding. The degree of inhibition did not increase with time (Fig. 1), and vesicle formation after fivefold dilution of the post-nuclear supernatant was still inhibited by GTP γ S (data not shown). If the formation of secretory vesicles were dependent on the continuous delivery of components to the *trans* Golgi network by fusion of other vesicles with this compartment, and if GTP γ S were to inhibit the fusion of these other vesicles, one would expect the degree of inhibition to increase with time and vesicle formation to be sensitive to dilution; neither was the case.

With respect to cell-free membrane traffic, only fusion events between vesicles (or donor compartments) and acceptor compartments have so far been reported to be inhibited by GTP γ S; this is thought to reflect the involvement of GTP-binding proteins

in the targeting of vesicles to, and their fusion with, acceptor membranes^{7-15,17,18}. Our results indicate that GTP hydrolysis is also required for the budding of vesicles (constitutive secretory vesicles and immature secretory granules) from a donor compartment (the *trans* Golgi network). By analogy with conclusions drawn from cell-free fusion assays, we interpret the requirement for GTP hydrolysis in the budding of secretory vesicles to reflect the involvement of a GTP-binding protein (or proteins) in this process. Thus, GTP-binding proteins seem to have a more general role in membrane traffic than previously assumed¹⁸. Furthermore, GTP-binding proteins may be involved in other vesicle budding processes than the ones studied here. □

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TABLE 2 Inhibition of cell-free post-Golgi secretory vesicle formation is specific for non-hydrolysable guanine nucleotides

	ISG		CSV	
	Budding efficiency	% Of control	Budding efficiency	% Of control
Experiment 1				
Control	32.6	100	14.9	100
GTP γ S (10 μ M)	19.8	61	8.9	60
Experiment 2				
Control	36.3	100	29.6	100
GMP-PNP (100 μ M)	19.1	53	16.4	55
Experiment 3				
Control	35.6	100	12.3	100
ATP γ S (50 μ M)	32.6	96	14.2	115

Cell-free formation of both immature secretory granules (ISG) and constitutive secretory vesicles (CSV) was performed as previously described¹⁶, except that samples were incubated in the absence or presence of 10 μ M GTP γ S, 100 μ M GMP-PNP and 50 μ M ATP γ S as indicated. After incubation at 37 °C for 60 min, the samples were analysed and the budding efficiencies calculated as described in Table 1. For each experiment, budding efficiencies are also expressed as per cent of control.

Lipid cell culture supplements

R.H. Goodwin

Essential lipid mixtures can substitute for vertebrate serum in the culture of invertebrate — and some vertebrate — cells, tissues, parasites and pathogens. The resulting membrane lipid modifications promote hormonal signalling.

SOME lipids of eukaryotic cellular membranes are tissue and species specific and change (1) with the state of differentiation, (2) when cells are directed to resume growth, as in tissue repair, or (3) when cells are directed to produce specific products¹⁻³, as in glandular secretion or virus replication. Such observations relate to the emergent understanding that polymorphic membrane lipid states can be linked to various structural configurations and functional processes^{4,5}. At the same time, normal cells demonstrate tissue-specific membrane sphingolipid⁶ and phospholipid molecular species asymmetry and specific associations with membrane proteins that are dependent upon both the phospholipid species and their specific content (ω -3 vs ω -6) of polyunsaturated fatty acids. These cellular states, and the resulting regulation of cellular functions, have been demonstrated by several improved methods that are able to distinguish such subtle processes^{7,8}.

Inevitably, similar critical invertebrate and microbial cell membrane lipid asymmetries, polymorphic lipid states and lipid growth requirements will also be defined, albeit more slowly⁹. The mycoplasma *Acholeplasma laidlawii* has served as a useful model in biochemical and biophysical lipid studies of the physical properties, structure and function of cellular membranes¹⁰. Moreover, a recent study of Semliki Forest virus apparently resolves earlier experimental confusions concerning the phospholipid asymmetries in enveloped viruses, which reflect the plasma membrane composition of their host cells¹¹. It is now accepted that the lipid-A portion of Gram-negative bacterial membranes carries most, if not all, of the endotoxic and other bioactivities exhibited by the bacterial lipopolysaccharide¹². Two recent malarial parasite lipid studies indicate both infected host cell phospholipid dependency and parasite phospholipid supply sensitivity^{13,14}.

While the requirement for specific extracellular matrix and hormonal components for the growth and differentiation of specific animal cell types *in vitro* is understood, it is not known whether there is a parallel requirement by these cells for specific lipids. As the preceding discussion and references suggest, the lipid content of native (*in vivo*) animal cell membranes is more specific and more

closely controlled than was formerly accepted. However, the delicate electrical membrane relationships that exist between lipids, proteins, proteoglycans, and other membrane-inserted components are extremely difficult to define experimentally. It is only recently that convincing studies have demonstrated the specific cell membrane asymmetries that develop between some polyunsaturated phospholipids in association with membrane proteins^{1,7,8,15}.

Until recently, tissue culture researchers relied heavily on transformed cell types, which have fewer essential lipid requirements than normal cells. Consequently, the topic of lipid specificity for cultured cells has generally been ignored¹⁶.

General lipid supplementation

Recently, manufacturers have made available sterol, lipoprotein, phospholipid, and hormone-containing extracts, supplements and proprietary media that contain better preserved and higher concentrations of certain vertebrate serum components than can be found in commercially available whole serum. Other supplements that are now available include animal glandular extracts, cell culture-derived hormones, and various hormone and growth factor complexes. Some of these products (Bovine cholesterol concentrate, ICN Biomedicals, Costa Mesa, California¹⁷; CPSR-1 and CPSR-2, Sigma, St Louis, Missouri¹⁸; EX-CYTE VLE, Miles, Kankakee, Illinois (W.F. Hink, personal communication) and similar extracts and combinations prepared by researchers¹⁹⁻²², have been used with reduced serum or serum-free media to decrease culturing costs, eliminate protein in the culture medium, or to define cultured invertebrate cell or parasite nutritional requirements.

Specific lipid supplementation

Lipids that are essential for parasite or invertebrate cell growth may be absent, suboptimal in concentration, or unavailable for effective use depending upon the method of *in vitro* presentation. Free or serum albumin-bound fatty acids do not closely mimic the natural *in vivo* lipoprotein presentation, which involves phospholipids associated with peptides and proteins.

Certain studies have shown, however,

that specific peptides and proteins may not be necessary for fully effective phospholipid supplementation *in vitro*^{23,24}. While certain unsaturated fatty acids (linoleate) can potentiate *in vitro* cell growth stimulation by hormonal factors, they are not by themselves able to stimulate growth. However, it has been noted that there is a qualitative difference between free essential fatty acid supplementation, which acts together with hormones as 'incomplete mitogen', and phospholipid essential fatty acid supplementation, which acts by itself as 'complete mitogen'²⁵. Similar *in vitro* stimulation associated with phospholipid supplementation has been noted for other mammalian cell types by Iscove²⁶ and others.

Certain insect cell culture systems have also used phospholipid essential fatty acid supplementation. While either a specific growth factor (glycyl-L-histidyl-L-lysine) or fatty acids and sterol provided as emulsions support the continuous culture of gypsy moth cell lines in serum-free media²⁷, only sterol-phospholipid peptoliposome supplementations support continuous culture of certain cotton bollworm cell lines in serum-free media²⁰. Furthermore, only sterol-phospholipid peptoliposome supplementation will support the continuous serial replication of baculoviruses²⁰ and an entomopoxvirus²⁸ in such media.

More recently, similar phospholipid supplementations have demonstrated the ability to prevent the usual decrease in virulence of baculoviruses serially propagated in cell cultures²⁹. These supplementations can, therefore, remove major impediments to the production in cell culture of insect viruses for microbial insect control, and may extend the application of the baculovirus vector system to include the production of more complex genetically engineered (lipo-) proteins. While these moth cell and virus supporting lipid supplementations emphasize the ω -3 polyunsaturates known to be required nutritionally by these (lepidopteran) insects⁹ and their (fibroblastoid) cell lines in culture, similar supplements may not be as applicable to other cell types, insect orders or invertebrate classes.

Some insects have been shown to contain mainly ω -6 fatty acids, particularly in their phospholipids (for example, adult

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American cockroach, Horse lubber grasshopper, Greater wax moth, Yellow mealworm, and the *Anopheles stephensi* mosquito³⁰. Although Rangeland grasshopper primary embryonic tissue cultures initially contained several subcultivable cell types, including epithelial cell sheets, all cell types other than fibroblasts and muscle cells were lost in early culture transfers when their medium was supplemented mainly with ω -3 fatty acid-containing sterol-phospholipid liposomes³¹. When certain ω -6 unsaturated fatty acids were substituted in the phospholipid preparation for most of the ω -3 fatty acids and the concentration of vertebrate serum was decreased or eliminated, early cell culture transfers of grasshopper embryonic cells retained increased ratios of epithelial and other non-fibroblastoid cell types. Further supplementation of these cultures with vertebrate hormone complexes (Mito-Plus, Collaborative Research, Bedford, Massachusetts, and P.Neurex, Upstate Biotechnology, Lake Placid, New York) and with cell culture grade bovine serum albumin (Miles), resulted in subcultures that contained mainly epithelioid cell types; of these, only the Mito-Plus supplemented, serum-free cultures were able to support the partial replication (viroplasms, nucleocapsids and spheroid protein production) of a fat body tissue-specific entomopoxvirus (R.H. Goodwin, unpublished observation).

Hormone supplementation

While several insect cell lines have been cultured in serum-free media lacking hormones, success has been limited because invertebrate cell growth factors have not been defined. At the same time, many invertebrate neuropeptides have demonstrated immunocytochemical cross reactions to active vertebrate peptides. An increasing number of these have been sequenced and demonstrate substantial homologies with their vertebrate counterparts^{32,33}. So far, only a few invertebrate cell cultures have demonstrated any response to vertebrate hormone complexes; further growth and/or dif-

ferentiation responses *in vitro* are likely to be dependent upon a better duplication of *in vivo*-specific cell membrane lipid composition^{9,30}. Lipid tissue analyses³⁰ of other invertebrate species (and certain fastidious vertebrate tissues) followed by incorporation of the relevant essential polyunsaturated phospholipids and sterols in nutrient peptolipid or proteolipid media supplements should greatly extend the range of invertebrate (and vertebrate) cells and tissues that can be cultured, while retaining significant tissue-specific functions, partial differentiation, and tissue resembling cellular associations. □

Ronald H. Goodwin is at the Rangeland Insect Laboratory, USDA/ARS, Montana State University, Bozeman, MT 59717, USA. For more information, fill in reader service number 100. Mention of proprietary names does not constitute endorsement by the USDA.

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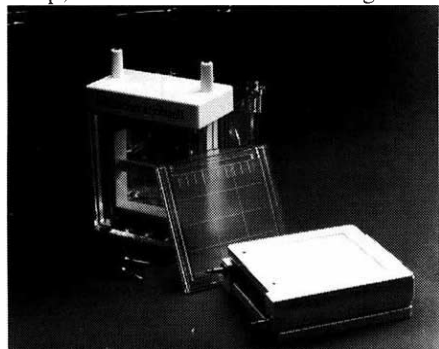
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Reader Service No.21

Coming up at Biotechnica '90

Over 500 exhibitors will be represented at next week's Biotechnica '90 to be held in Hannover, West Germany. Featured exhibits will include a primer analysis software package and a hollow-fibre bioreactor for monoclonal antibody production.

WHEN choosing a gel for the fractionation and purification of low molecular weight nucleic acids, Schleicher and Schuell recommends its **pre-cast tris borate EDTA polyacrylamide mini-gels** (*Reader Service No. 101*). Other uses include the high-resolution separation of products of enzymatic amplification by *Taq* DNA polymerase and the purification of amplification products from their oligonucleotide reaction primers. The pre-cast mini-gels provide an alternative to agarose gels and are designed to produce sharp, clear bands with low background



Pre-cast TBE polyacrylamide mini-gels from S & S — see stand F14.

staining. The mini-gels are used in conjunction with the S & S mini-vertical electrophoresis system for gel fractionation and electrotransfer. Using this set-up, two gels can be run simultaneously, with each process taking less than one hour, says S & S. Pre-cast tris glycerine gels are also available for protein electrophoresis in a variety of isocratic and gradient formats. See stand F14.

MedProbe of Norway, the European distributor for National Biosciences, is selling version 3.4 of a **primer analysis program** called OLIGO (*Reader Service No. 102*). OLIGO helps researchers select the right DNA oligonucleotides, that is, oligonucleotides that are not self-complementary, do not form duplexes with themselves or any other selected sequences, and hybridize only to the specific target DNA sequence. In addition, by using advanced chemical algorithms, OLIGO can be used to calculate the optimal annealing/hybridizing temperatures for the test conditions. MedProbe says the program is particularly useful for selecting pairs of oligonucleotides for polymerase chain reaction analysis. OLIGO is compatible with various file formats, including EMBL, GenBank and other ASCII

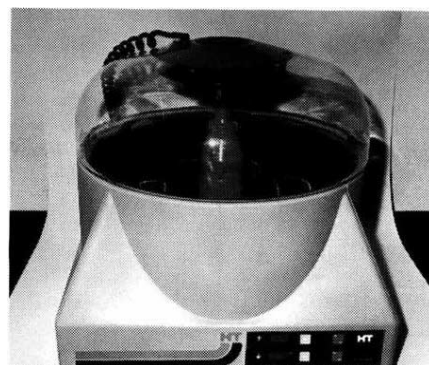
text files. A program for IBM PC/AT and compatible computers is available for \$440 (US) (non-profit organizations). MedProbe says a Macintosh version will be available later this year. OLIGO will be on display at Biotechnica '90 on stand G14.

Genzomed Biotech will be exhibiting its new **sial HPLC column** for the analysis and separation of different sialic acids and intermediates in the sialic acid metabolic pathway on the Maryland, USA stand, number D38 (*Reader Service No. 103*). The column can, says Genzomed, be used both for the analysis of sialic acids with picomole sensitivity and enzyme assays — sialidase, *N*-acetylneuraminidase lyase, CMP-sialic acid synthase, CMP-sialic acid hydrolase and sialate-*O*-acetyltransferase. Separation of sialic acids can be achieved in less than 10 minutes at neutral pH, says the company.

Sera-Lab, exhibiting on stand A27, has a new anti-human **monoclonal antibody reagent specific for terminal deoxynucleotidyl transferase (Tdt)** which is designed for flow cytometry applications (*Reader Service No. 104*). The company will supply the anti-human Tdt reagent, which comprises a cocktail of three different mouse mAbs, labelled with FITC or in an unlabelled form. Tdt is a nuclear maturity marker used in immunophenotyping acute lymphoblastic and myeloblastic leukaemias, but is most informative when used in combination with markers for membrane antigens. Anti-human Tdt can facilitate this simultaneous analysis, says Sera-Lab.

Culture conveniences

Infors, exhibiting on stand D67, has introduced the Aerotron **incubator shaker**, which was developed as a direct modification of the company's Aquatron waterbath shaker (*Reader Service No. 105*). The Aerotron's acrylic cover is equipped with a built-in heater/air circulation system that is designed to provide even temperature control from 5°C above ambient to 60°C with an accuracy of $\pm 0.2^\circ\text{C}$, says Infors. The incubator shaker has a silent magnetic drive and can reach shaking speeds of between 20 and 400 r.p.m. The large-capacity chamber can hold fifteen 250-ml tubes or six 1,000-ml flasks. Optional fittings are available for test-tube racks. Built-in connections



Aerotron: Infors' shaker has in-built temperature control.

allow overgassing with air/CO₂, and a safety switch ensures that the circulator is off whenever the lid is opened.

The Tecnoroll **roller bottle system** from Tecnomara provides optimal growth conditions for the mass production of viruses and cells (*Reader Service No. 106*). The gentle rotating action of the bottles is, says Tecnomara, ideal for exposing wall-adhesive cultures to nutrient media and gas. Cultures produced using the Tecnoroll system can be grown at higher cell densities and with minimum quantities of nutrient media, says the company. The apparatus can accommodate between 5 and 90 roller bottles, providing up to 70,000 metres of growth surface. Tecnomara's full product range will on display on stand G10.

ICN Biomedicals has introduced the RapidCell system, which consists of a series of **microcarrier beads** for use in cell cultures that require a solid support (*Reader Service No. 107*). The RapidCell series includes collagen-coated, glass and plastic beads, each designed for specific



RapidCell microcarrier beads — solid supports for cell growth.

applications. Use of the beads promotes rapid cell growth and allows cells to be grown at higher densities, says ICN — see stand 21.

According to Amicon, its Mini Flo-Path ready-to-use **hollow-fibre bioreactor** provides an ideal replacement for mouse ascites in the production of monoclonal antibodies (mAbs) in research quantities (*Reader Service No. 108*). With the Flo-Path mini-bioreactor, mammalian cells grow on or around the exterior of artificial capillaries, which are designed to promote high rates of nutrient supply and waste removal. Used as a replacement for 20 to 40 mice, the bioreactor can produce 5–50 mg of mAb per day, with up to 95 per cent purity and at a concentration of 1–10 mg ml⁻¹, says Amicon. The system can be used continuously for 60–90 days, with between three and five harvests per week. The \$250 (US) Mini Flo-Path system is supplied pre-sterilized and ready for use. See Amicon on stand B25.

LH Fermentation will be using Biotechnica '90 to launch its new range of **measurement and control modules**, which are compatible with most commercial fermentors (*Reader Service No. 109*). Five modules are available for monitoring agitation speed, dissolved oxygen, pH/redox, temperature and anti-foam. See the measurement modules and LH Fermentation's benchtop and free-standing bioreactors for microbial or animal cell applications on stand A13.

New lines in labware

If you are looking for a **refrigerated benchtop centrifuge** for high-speed applications with up to 28,000 r.p.m., then the Biofuge 28RS from Heraeus may provide



Biofuge 28RS — spin control to 28,000 r.p.m. the answer (*Reader Service No. 110*). The 28RS is supplied with a range of microprocessor-controlled centrifugation programs that are designed to solve complex separation problems, such as the quantitative pelleting of proteins, DNA/RNA and antibodies, preparation of samples for

HPLC/FPLC analysis and biochemical assays. Depending upon the rotor, and users can choose from a selection of microtube, swing-out or continuous-flow rotors, the 28RS has a maximum capacity of 80 × 1.5 ml or 6 × 94 ml. When the continuous-flow rotor conversion kit is used to perform the continuous separation of liquid and solid mixtures, the 28RS can handle sedimentation volumes of 300 ml. Other design features include refrigeration control between 0 and 40 °C, a micro-computer system that offers the user 32 programs to ensure consistent run conditions, automatic rotor identification, and nine acceleration and braking routines. See Heraeus on stand C26.

The DSM 962 **scanning electron microscope (SEM)** from Carl Zeiss is the latest instrument in a family of fully digitized SEMs that, in addition to its capabilities as an analytical instrument with a large specimen chamber, offers a dedicated image processor with applications software (*Reader Service No. 111*). The DSM



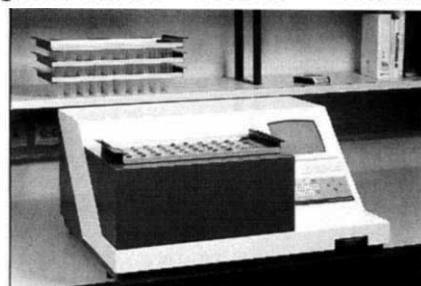
Illuminating ideas for SEMs from Zeiss.

962's integrated digital framestore allows a permanent image display in the TV mode in normal room lighting. The real-time image processing and analysis capabilities are interactively controlled using an integrated menu monitor, keyboard and mouse. The short doubly-shielded electron optical column with its three independently controlled lenses provides, says Zeiss, both optimum performance at all operating voltages and a current stability only achieved in dedicated microprobes. A turbomolecular pump, which is fitted as standard, creates a contamination-free specimen environment. Zeiss will on stand H15.

The Model 840 **amino acid analyser** from Knauer combines the reproducibility obtained with postcolumn derivatization with the speed and sensitivity of HPLC (*Reader Service No. 112*). Amino acids are separated on a cation-exchange column and then derivatized with the fluorescent reagent OPA, which identifies only primary amino acids: secondary amino acids must be converted with hypochlorite

before they will react with OPA. Both conversion and derivatization take place in the 'knitted' and inert hypochlorite and OPA reactors. Fluorescent amino acid derivatives are detected by a detector with sensitivity in the low picomole range. The modular amino acid analyser system will be on display on stand F22.

For high-throughput laboratories carrying out radioimmunoassays, Pharmacia Wallac Oy has designed a manual **gamma counter** — the 1460 — which has



High-throughput gamma counting.

the capacity to count up to 50 samples simultaneously (*Reader Service No. 113*). The instrument has 50 detectors and features a 'dynamic normalization' facility that eliminates the effects of detector imbalance and compensates for any 'drift' in the performance of a detector, whether it be caused by aging, voltage fluctuation or environmental instability, says Pharmacia. The counter can be used either with a PC and Pharmacia's MultiCalc data collection and analysis software, or it can be controlled by a sample-handling robot to form an automated system. Pharmacia will be on stand B38.

Ultrasette, the tangential flow **filtration device** from Filtron, can be used for ultrafiltration (UF) and microfiltration (MF) of volumes in the range of 200 ml to 5 litres (*Reader Service No. 114*). As the device features complete encapsulation of the membrane cassette inside a plastic outer coating, it is, says Filtron, particularly useful for processing pathogenic and biohazardous materials. Ultrasette can be used for the concentration, purification, desalting and washing of macromolecular solutions and cell broths. It can also be used for continuous and batch depyrogenation. Three types of filter unit are available: the alpha series with an anti-foam resistant polyethersulphone (PES) UF membrane; omega series with a low protein binding PES UF and MF membrane; and the fluoro series with a polyvinylidene difluoride membrane for cell harvest applications. See stand H14. □

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